GENE EXPRESSION IN B AND T LYMPHOCYTES:

(1) Evolution of Rat C_K Alleles

(2) The T-cell Receptor Problem

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This thesis is dedicated to my
parents, self-taught, who said, "Son, get
an education."

The most mysterious aspect of difficult science is the way it is done. Not the routine, not just the fitting together of things that no one had guessed at fitting, not the making of connections; these are merely the workaday details, the methods of operating. They are interesting, but not as fascinating as the central mystery, which is that we do it at all, and that we do it under such compulsion.

- Lewis Thomas

Not just a job, it's an adventure.

Army Recruitment Commercial

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ABSTRACT

The amino acid sequence of the two kappa chain constant region allotypes found in inbred rat strains indicated that these alleles are very different and therefore may have had an unusual evolutionary history. To understand the evolution of these genes, serologic tests were performed to determine if inbred rats express latent or unexpected C_κ alleles. They apparently do not do so. Wild Norway rats were tested, and it was found that the laboratory strains do not represent a subset of the rat C_κ polymorphism. Further tests indicated that only one of the two serologic specificities could be found in related rodent species.

The structure of the T cell antigen-binding receptor is a major controversial issue in immunology. It has been asserted that the T cell antigen-receptor is homologous to immunoglobulins, and one popular theory contends that V_H genes are responsible for the specificity of the receptor. We tested these theories by hybridizing immunoglobulin DNA probes to RNA and DNA from cloned T cells. First, we determined that the C_λ, J_κ, C_κ, J_H, C_μ and C_α genes and the sequences involved in heavy chain class switching are not rearranged in a T helper, a cytotoxic T cell and a T lymphoma. These cells also do not transcribe C_κ, C_λ, J_H, C_μ and C_α RNA. Second, a cDNA clone encoding heavy chain variable region characteristic of most B cells which respond to the antigen GAT was isolated and sequenced. Poly(A)$^+$ RNA was prepared from 12 cloned T lymphocytes specific for GAT. While six of these T cells display antigenic determinants present on immunoglobulins that bind GAT, none of them contained a transcript homologous to the cDNA probe. Finally, using a random primer, large cDNA libraries (10^5-10^6 colonies) were constructed from three T-cell hybridomas. These libraries were screened by two separate, well-characterized methods which should permit the detection of all or most V_H gene segments. No V_H cDNA colonies were found by these methods. Therefore immunoglobulin gene segments are not likely to be part of the T cell antigen receptor.

The I-J serologic specificity has been reported to be present on T cell-derived antigen-binding molecules. Cosmid clones have been previously obtained containing all the sequences between the I-A and I-E subregions of the murine major histocompatibility complex, where I-J has been genetically mapped. The putative I-J DNA does not, however, hybridize to RNA from I-J positive suppressor T cells. Also, suppressor T lymphocytes do not rearrange this DNA. Therefore the I-J coding sequences must map elsewhere.

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Chapter 1

EXPRESSION AND EVOLUTION OF RAT KAPPA CHAIN CONSTANT REGION ALLOTYPES

Introduction

Immunoglobulin kappa light chains from inbred laboratory rat (Rattus norvegicus) strains have two serologically detectable forms that segregate as codominant alleles (Armerding, 1971; Gutman and Weissman, 1971; Rokhlin et al., 1971). The genetic locus that controls the expression of these markers is RI-1, and the corresponding alleles are RI-1^a and RI-1^b. The biochemical differences responsible for these serological markers are located in the constant region of the kappa chain (Nezlin et al., 1974). Eighty-one out of 111 residues in the constant regions of the RI-1^a and RI-1^b kappa chains have been compared by amino acid sequence analysis (Gutman et al., 1975) and it was found that they differ by 10 amino acid substitutions and a sequence gap.

Allelic variants at a single genetic locus generally differ by one or a few amino acid substitutions. For example, over 200 human hemoglobins have been examined and most differ by one residue, a few differ by two and only one differs by as many as three residues (Hunt et al., 1972). The use of the term "complex allotypes" has been suggested to denote serologically detectable markers which segregate as Mendelian alleles at a single genetic locus, and differ from one another by multiple amino acid substitutions (Gutman et al., 1975; Farnsworth et al., 1976; Silver and Hood, 1976). On this basis, rat kappa chain alleles, rabbit kappa chain constant region (Farnsworth et al., 1976), and heavy chain variable region (Strosberg, 1977) allotypes, and H-2K and H-2D alleles of the murine major histocompatibility complex (Silver and Hood, 1976; Maizels et al., 1979) have been classified as complex allotypes. The term complex allotypes is purely descriptive of the multiple differences, but is meant to focus attention on the fact that these alleles may have had an unusual evolutionary history.

There are three general models which explain the evolution of complex allotypes (Fig. 1; Gutman et al., 1975): 1) The alleles evolve in a normal fashion from

a single ancestral gene by the successive occurrence and fixation of multiple mutations in this gene. At many of the loci encoding complex allotypes, one must postulate either intense selective pressures to account for an unusually rapid divergence of the alleles, or that the alleles began to diverge long before the formation of the present-day species. 2) An early gene duplication may allow the fixation of many nucleotide differences in these duplicated genes. Two homologous but unequal crossovers could then generate two different populations, each one having only one of the duplicated gene copies. 3) The allelic genes are actually duplicated genes present on the same homologue and a control mechanism selects which of the pseudoallelic forms is expressed. In this case, the genetic studies are detecting allelic forms of a control mechanism rather than alleles for the structural genes.

We have studied the rat kappa chain constant region (C_K) allotypes in order to better understand the evolution of complex allotypes and to distinguish between the three models for the formation of complex allotypes listed above. Specifically, we tried to answer three questions: 1) Do inbred rat strains express latent allotypes? The transient and/or low level expression of allotypes not expected according to the conventional Mendelian behavior of alleles (i.e., two or more allotypes in an inbred rat strain) would provide evidence for a gene duplication and a control mechanism regulating expression of the duplicated genes. We employed a sensitive, quantitative radioimmunoassay (RIA) in order to detect unexpected RI-1 alleles (latent allotypes). 2) How many RI-1 alleles are present in Rattus norvegicus? The detection and characterization of new C_K allotypes would help in understanding the evolutionary history of these alleles. For example, the detection of cross-reactive light chains "intermediate" between the RI-1^a and RI-1^b specificities would tend to support the hypothesis that the alleles diverged in a normal fashion from a single ancestral gene in Rattus. To detect such alleles, we tested wild Rattus norvegicus with anti-allotype sera. (3) When did the RI-1 alleles begin to diverge? Could such divergent alleles

have been formed from a single ancestral gene in Rattus norvegicus? To address this point, we tested three other rodent species for the presence of RI-1^a and RI-1^b kappa chains.

Materials and Methods

Inbred rats. WF/fMai, BN/fMai, LEW/fMai, ACI/fMai and F344/fMai were originally obtained from Microbiological Associates, Walkersville, Maryland, and were bred for several generations in our colony.

Wild rats. Rattus norvegicus (Norway rats) were trapped at Los Angeles and Seal Beach, California, with the help of Orange County Vector Control. Dr. David Gasser, University of Pennsylvania, Philadelphia, Pennsylvania, kindly sent us the sera from eight wild Norway rats trapped in Philadelphia. Neotoma fuscipes (dusky-footed wood rats) were trapped in the Santa Monica mountains of Los Angeles, California. Rattus rattus (roof rats) were trapped in Los Angeles, California, and the sera from these rats was the gift of Dr. John Estes, University of Southern California Medical School, Los Angeles, California.

Preparation of anti-allotype sera. Anti-RI-1^a was made in WF rats against ACI immunoglobulin and anti-RI-1^b was made in ACI rats against WF immunoglobulin. The anti-allotype sera were prepared according to published procedures (Gutman and Weissman, 1971). Briefly, rats were given two or three intraperitoneal injections of pertussis vaccine (Eli Lilly and Co., Indianapolis, Ind.) and bled one week later. Anti-pertussis rat serum was mixed with the vaccine and the pertussis-anti-pertussis complex served as the immunogen.

Radioimmunoassay. A solid phase radioimmunoassay (RIA) was used to quantitate allotype. The globulin fraction of the anti-allotype serum was coupled to CNBr-activated Sepharose (March et al., 1974) at a ratio of approximately 1 mg of protein per ml of packed beads. Radiolabeled antigens were the IgG fraction of WF

or ACI serum proteins. These were prepared by precipitating pooled WF or ACI sera three times with 18% (w/v) Na_2SO_4. The precipitate was then filtered through a 2.5 x 100 cm Sephadex G-200 (Pharmacia, Uppsala, Sweden) column in BBS (0.17 M H_3BO_3, 0.13 M NaCl, 28 mM NaOH, pH 8.1 with 0.02% NaN_3). Gel electrophoresis in 0.1% SDS under reducing conditions (Fairbanks et al., 1970) indicated that the peak containing IgG which eluted from the G-200 column consisted of >90% heavy and light chains. Immunoglobulin antigens were iodinated by the chloramine-T method (Hunter, 1973). One hundred to 150 μCi of ^{125}Iodine was added to 5 μg of protein and gave a final specific activity of 5-20 μCi/μg. To test antigen binding, 4 ng of ^{125}I-labeled antigen and Sepharose coupled anti-allotype were placed in a Beckman microfuge tube. PBS (0.05 M phosphate, pH 7.4, 0.15 M NaCl) with 10% fetal bovine serum (FBS) was added to bring the final volume to 350 μl and the contents were rotated continuously at room temperature in an end-over-end mixer. To obtain maximal sensitivity for inhibition assays, the labeled antigen was used in slight excess (about 50% of added cpm bound) to the anti-allotype reagent. Antigen binding under these conditions reached a plateau at 12 hours. We mixed the tubes for 18 hours, spun them down in a Beckman microfuge, and removed the supernatant. The pellet containing anti-allotype coupled to Sepharose and any bound labeled antigen was washed twice with 350 μl of PBS containing 10% FBS. The final pellet and the total supernatant volume were counted in a Chicago-Nuclear γ counter. All experiments included a blank tube which contained ^{125}Iodine-labeled antigen, underivatized Sepharose and PBS with 10% FBS in a total volume of 350 μl. The fraction of the radiolabeled antigen bound to the antiserum was calculated according to the following formula:

$$\text{Fraction bound} = \frac{\text{cpm pellet}}{\text{cpm supernatant}} - \frac{\text{cpm pellet blank}}{\text{cpm supernatant blank}}$$
$$\text{cpm pellet} + \qquad \text{cpm pellet blank} +$$

Standard curves were constructed using various amounts of the purified unlabeled IgG

fractions (described above) as competitors for binding with the ^{125}I-labeled antigen. Protein concentrations were estimated by reading the optical density at 280 nm (1.4 O.D. = 1 mg/ml).

$$\% \text{ inhibition} = (1 - \frac{B_i}{B_o}) 100$$

B_i = fraction bound with inhibitor concentration i

B_o = fraction bound without inhibitor

Inhibitors were mixed for 1 hr with the anti-allotype reagent before addition of labeled antigen. Aside from standard curves, unfractionated serum samples were used as inhibitors. All data points were taken in triplicate. Inhibition of binding greater than or equal to 10% was considered significant.

Passive hemagglutination. Passive hemagglutination and inhibition of passive hemagglutination were carried out as described (Gutman and Weissman, 1971). WF and ACI rat anti-sheep red blood cell antisera were prepared and these sensitizing antisera were mixed with sheep red blood cells at a dilution just past the agglutination end point. Sheep red blood cells coated with rat immunoglobulin were then tested with 20 µl of anti-allotype antibody and serial dilutions of inhibitor sera.

Allotype suppression. One-day-old rats received an intraperitoneal injection of 0.2 ml of anti-allotype sera. The process was repeated on day seven.

Results

Rat alloantisera recognize light chain allotypes. In most cases we used a radioimmunoassay for serologic tests. This assay was more sensitive, quantitative and reproducible than the hemagglutination assay that had been previously employed. The RIA should therefore more easily permit detection of low levels of latent allotype expression and is better suited to distinguish cross-reactive from identical light chain serotypes.

Figure 2 shows examples of standard curves obtained using Sepharose-coupled antisera. The AVN, BN, LEW and LOU strains are reported to express the RI-1^b

specificity (Gasser, 1977). Two µl of pooled serum from each of these strains inhibited binding of ^{125}I-WF Ig (RI-1^b) to antibodies against WF Ig. Binding of ^{125}I-ACI Ig (RI-1^a) to antisera against ACI Ig was not inhibited (data not shown). The DA, F344 and OM strains reported to be RI-1^a positive (Gasser, 1977) inhibited binding of ^{125}I-ACI Ig but not binding of radioactive WF Ig. Isolated ACI and WF light chains can completely inhibit binding of radioactive whole immunoglobulin by the appropriate antisera (data not shown). These results indicate that our antisera recognize the defined light chain allotypic specificities.

Latent allotype expression in normal rats. Inbred laboratory rats were tested for latent allotype expression. Initially, 31 rats were bled by cardiac puncture and their whole sera were individually tested. For example, serum from an RI-1^a homozygous rat was added to anti-allotype (anti-RI-1^b) and labeled RI-1^b immunoglobulin and the percent inhibition of antigen binding was observed. We deliberately chose to test rats of varied backgrounds since a control mechanism might "leak" or permit latent allotype expression only under limited circumstances (Strossberg, 1977). Our test sera came from rats from five different inbred strains, both male and female, ranging in age from three weeks to over one year old. Six animals had been repeatedly immunized with pertussis vaccine.

Fifty µl of serum from 19 RI-1^b homozygotes were tested with anti-RI-1^a serum. This is at least 250 times as much as was needed to completely inhibit binding of labeled RI-1^b immunoglobulin to anti-RI-1^b sera (data not shown). Eighteen out of 19 RI-1^b sera inhibited binding of labeled RI-1^a immunoglobulin, with values ranging from 12.9% to 42.9% (Table I). For the RI-1^a rats, 50 µl of sera from 10 out of 12 animals inhibited binding of labeled RI-1^b, ranging from 14.0% to 59.3% inhibition (Table I). Fifty µl of pooled BALB/c mouse serum did not cause inhibition of binding of anti-allotype sera to either RI-1^b or RI-1^a rat immunoglobulin. Thus, the inhibition observed is not an artifact caused by having a large amount of serum

protein present in the assay tube.

The mean inhibition of binding of the anti-allotype sera to RI-1^a immunoglobulin by 19 RI-1^b sera was 28.6%. The mean inhibition of binding of RI-1^b by 10 RI-1^a sera was 34.3%. To rigorously establish a serological identity between the cross-reactive material and the unexpected or latent C_κ allotype, we should be able to satisfy two criteria: 1) the cross-reactive material should be able to inhibit at least 90% of the binding of the Sepharose-coupled anti-allotype to the labeled immunoglobulin; and 2) the slope of the inhibition curve should be similar to the slope of a standard curve using purified immunoglobulin with the allotype in question. In order to increase the amount of competitor serum added to the assay tubes by an order of magnitude, we concentrated the sera from single bleedings of 12 individual rats by pressure dialysis. Most of these rats had been tested earlier using 50 µl of serum (Table I). Inhibition curves for four of these serum samples are shown (Fig. 2). The data fall into two groups. 1) In 10 cases, the competitor appears to only partially cross react with the unexpected allotype. The percent inhibition in these cases (two are shown in Fig. 2) either increases very slowly, reaches a plateau, or even decreases with increasing concentration of serum. 2) In two cases, rats WF 10 and AVN 02 (Fig. 2B), the inhibition points fall on a straight line. Neither line contains many points since the experiment in Figure 2B required a total of 2.4 ml of AVN and 3.2 ml of WF serum. For the AVN 02 serum, the maximum inhibition was 67% and for the WF 10 serum the maximum was only 51% and so it is possible that we are observing plateau values.

Allotype suppression. We attempted to suppress the nominal allotype of RI-1 homozygous rats reasoning that latent C_κ allotype production might compensate for the suppressed allele of the homozygote. Our first step was to show that C_κ allotypes could be suppressed in rats.

Neonatal F_1 rats which had received two injections of antiserum to their paternal C_κ allotype show a profound depression in the synthesis of that allotype six weeks later (Table II). The capacity to be suppressed does not seem to be restricted to either allotype or to any particular strain combination of the ones tested. Two animals assayed at four months of age still showed only about 1% of normal C_κ allotype synthesis. Allotype suppression has also been characterized in mice and rabbits (Herzenberg et al., 1975; Mage, 1975). It is possible that allotype suppressed F_1 ($1^a x 1^b$) females could be backcrossed to 1^a homozygous males in order to obtain allotype suppressed homozygous progeny. Unfortunately, disease in our colony wiped out the few suppressed female rats we had obtained and this experiment was not completed.

Allotype suppression was attempted by directly injecting anti-RI-1 sera into normal homozygous neonates. Concentrated sera were used, and these animals received four times the dose of the F_1 rats. This treatment apparently had little effect on the expression of the expected C_κ allotype of the homozygous animals which in all cases except one was greater than 2 mg/ml of serum (data not shown). A small amount of latent allotype expression observed in these animals can be attributed to the persistence of the injected anti-allotype immunoglobulin in their circulation. After a few weeks the observed levels of cross-reactive material were no greater than those seen in normal homozygotes (data not shown).

Number of RI-1 alleles in Rattus norvegicus. Additional alleles might exist in Rattus norvegicus but may not have been detected because the inbred strains represent only a portion of the total polymorphism present in the species. Many inbred rat strains share a common ancestry, apparently having originated from the early Wistar and Sprague-Dawley colonies (Palm and Black, 1971). We tested the sera of 24 wild Norway rats trapped in widely separated locales by inhibition of passive hemagglutination and in 17 cases by radioimmunoassay as well. As shown in Table III,

there are no rats lacking both allotypes, and the RIA data are consistent with there being no intermediate or partially cross-reactive forms (data not shown).

Interspecies relationships of RI-1 alleles. Table IV shows the inhibition of passive hemagglutination and RIA data obtained from other wild rodents that were trapped locally. Rattus rattus (family muridae) which is closely related to the Norway rat but does not interbreed with it (Robinson, 1965) has the RI-1^b but not the RI-1^a serologic specificity. On the other hand, the kappa chains of the BALB/c inbred mouse strain (also family muridae) show no cross-reaction with either anti-allotype. The New World rodent Neotoma fuscipes (family cricetidae) appears to share few specificities with any rat kappa chains, although a slight cross-reaction with RI-1^b may be just at the limit of detection.

Discussion

Complex allotypes are encoded by the mouse major histocompatibility complex (MHC), rabbit kappa chain constant region and heavy chain variable region loci, and by the rat kappa chain constant region locus. Several systems that exhibit multiple serological specificities such as the mouse IgG2a locus (Lieberman, 1978) and the human IgG3 locus (Fudenberg et al., 1978) may turn out to encode complex allotypes as defined by amino acid sequence criteria. Thus, complex allotypes constitute a major class of the polymorphisms found in the immune system and possibly in other systems as well (Gutman et al., 1975). We have studied the rat C_κ allotypes as a model system for understanding the evolution of complex allotypes.

Models for the evolution of complex allotypes

Three general models (Fig. 1) have been proposed for the evolution of complex allotypes.

(1) The alleles evolve in a normal fashion from a single ancestral gene by the successive occurrence and fixation of multiple mutations in this gene. In most cases

it is not easy to see how these alleles could have evolved from a single ancestral gene present in a species. A striking example is the b4 and b9 kappa chain constant region alleles of the rabbit which differ by 33 residues (Farnsworth et al., 1976). The observed sequence divergence is consistent with the alleles having been separate for over 50×10^6 years (Dayhoff, 1969). A second possibility consistent with this model is that the alleles began to evolve in a normal fashion long before the formation of the species that expresses them. New species formed in this evolutionary line must therefore have started from individuals already polymorphic at this locus.

(2) Complex allotypes may evolve by gene duplication and subsequent gene loss through homologous but unequal crossing over The gene duplication may occur before or after the formation of the species in which the particular polymorphism is found. The rate of fixation of mutations may be increased if we assume either that one gene is silent and can accumulate mutation free of stringent selective pressure or alternatively if both genes are expressed but only one functional gene is required for survival fitness. Later in evolution, two unequal but homologous crossing-over events can occur to generate two populations of organisms, each having a different form of the gene in question.

(3) Complex allotypes may evolve by gene duplication and be differentially expressed via a control mechanism. This model is similar to the second model in postulating a gene duplication which permits a rapid divergence of the duplicated genes. The critical difference in the second and third models is the arrangement and expression of genes in the contemporary organism. In the second model, allelic behavior for the duplicated genes is caused by a crossover event leading to a gene reduction while in the third model a control mechanism allows expression of only one of the gene copies. Similar control mechanism proposals have been made by others (Rivat et al., 1970; Bodmer, 1973). The regulatory or control gene model for the evolution of complex allotypes is unattractive since it postulates control which by an

unknown mechanism must mimic allelic inheritance. There are, however, at least two sets of genes in eukaryotes, those coded by the yeast mating type (Hicks and Herskowitz, 1977) and mouse TL loci (Old and Stockert, 1977) which involve control genes that mimic allelic inheritance.

Latent C_κ allotype expression is not observed in rats

One way to distinguish between the models listed above would be to find expression of unexpected or latent allotypes in inbred strains of rats.

We examined a total of 61 rats and six pools of sera (Tables I, II, Fig. 3, data not shown) for expression of latent allotypes. In many cases, the competitor serum gave a cross-reaction rather than a reaction of identity with immunoglobulin bearing the unexpected RI-1 allotype. In no instance could the rat sera inhibit more than 67% of binding of immunoglobulin with latent allotype and in most cases the inhibition curve appeared to plateau at about 60%. The structure of this cross-reactive material is unknown and we have no evidence that it is a κ light chain. It should be noted that the immunogen used to make the antisera is an antibody-antigen complex which could contain many different proteins. Precedents exist for puzzling, apparently artifactual cross-reactions of specific antisera with allotypic markers (Litwin, 1971; Mage et al., 1977). In two cases, probably because of a shortage of serum, plateau values for inhibition were not reached. Because of the widespread appearance of RI-1 cross-reactive material, we have assumed that the molecule causing inhibition in these cases is also cross-reactive rather than identical. However, if the inhibitor is the latent RI-1 allele, then AVN 02 expresses about 190 ng/ml of immunoglobulin with the $RI\text{-}1^b$ kappa chain and WF 10 expresses about 60 ng/ml of $RI\text{-}1^a$. These values are less than 0.01% of the concentration of the expressed or nominal allotype in the serum. While we have found no convincing evidence for the synthesis of latent RI-1 alleles, this does not entirely disprove the control mechanism model since such a hypothetical mechanism might not permit even low levels of latent allotype expression.

Our attempts at immunological manipulation of latent allotype levels were unsuccessful. In the course of doing these experiments, we were able to demonstrate nearly complete and chronic suppression of the paternal C_κ allotypes of F_1 rats (Table V). The attempt at direct suppression of homozygous rats failed despite the extra dose of antisera, presumably because of the large amount of maternally derived antibody present in the circulation of the neonate. However, suppression of κ chain allotype in homozygous rabbits, produced by backcrossing allotype suppressed F_1 females to normal homozygous males, led to elevation of C_λ rather than latent C_κ allotype expression (Mage, 1975).

Rattus norvegicus expresses only two RI-1 alleles

Expression of a kappa light chain which is either partially cross-reactive or does not react with the anti-allotype sera would shed light on the evolution of these alleles. For the first model, which postulates no gene duplication, we might expect a C_κ allele intermediate between the "extremes" of RI-1^a and RI-1^b to have been fixed in some populations. The detection of such an allele, would not, however, eliminate the other models. A large number of inbred rat strains have been typed for RI-1 alleles. Forty-four strains have the RI-1^b allele, and 16 have the RI-1^a allele. No strains have both allotypic markers, neither do any strains lack both these markers. There is no evidence for a third allele or a partially cross-reactive intermediate allele. Including the work reported here, five different sets of antisera give apparently equivalent results (Armerding, 1971; Gutman and Weissman, 1971; Beckers et al., 1974; Rokhlin and Nezlin, 1974; Gutman, 1977; Gasser, 1977). However, because many inbred rat strains originated from the same two colonies, these strains may not represent the full extent of Rattus norvegicus polymorphism. Therefore, a screening of wild Norway rats was undertaken. These animals typed as either RI-1^a, RI-1^b, or $1^a/1^b$ heterozygotes with no double negatives observed. Similar results have been reported by others (Nezlin and Rokhlin, 1976; Gutman,

1977), and it therefore appears unlikely that we have sampled only a very limited portion of Rattus norvegicus RI-1 polymorphism.

It is possible, however, that a low frequency allele which is not cross-reactive has escaped detection. For such an allele, the tests conducted will detect only x/x homozygotes, whereas a/x or b/x heterozygotes would type as RI-1 homozygotes. It also is possible that the anti-allotype sera recognize one or few immunodominant determinants among many differences in the constant region and that light chains assigned a particular RI-1 allele are really not identical to one another. Only a biochemical analysis of rat kappa chain constant regions can settle this question.

RI-1 alleles evolved before formation of Rattus norvegicus as a separate species

The Rattus rattus we tested had the RI-1^b, but not the RI-1^a allotypic specificities. This result is consistent with the data of George Gutman and his collaborators (Gutman and Moriwaki, 1979) who found the RI-1^b specificity is widely distributed among Rattus species. Thus it is clear that at least one of these alleles had evolved before the formation of Rattus norvegicus as a separate species. There is no evidence for any light chain identical to or cross-reactive with RI-1^a, and the origin of this specificity is mysterious. The New World rodent Neotoma fuscipes and a Mus musculus inbred strain displayed neither serotype.

Postscript - 1982

The rapid evolution of some immunoglobulin constant region allotypes is still poorly understood. Analysis of the rat C_K genes in the laboratory of Dr. George Gutman has proven that inbred rats expressing the RI-1^a or RI-1^b specificities contain a single C_K gene (Sheppard and Gutman, 1981). Thus, in this species the control gene model cannot be correct. Alleles of the mouse γ2a heavy chain constant region differ by 111 out of 1093 nucleotides compared (Schreier et al., 1981). A control gene model for these alleles is also not possible. Some of the changes present

in one γ2a allele may have been caused by a gene conversion event involving γ2b sequences. The situation for the rabbit C_κ locus is quite different. Amino acid sequence analysis has confirmed one case of latent allotype expression which was originally identified serologically (Yarmush et al., 1980). Southern blot hybridizations of a κ probe (b4) to DNA from b4, b5, b6 and b9 homozygous rabbits indicate that each rabbit contains multiple C_κ genes (Heidmann and Rougeon, 1982). It has not yet been determined if some of these hybridizing sequences are silent versions of expressed alleles, nor do we understand how the expression of the rabbit C_κ genes is regulated. Finally, it is not clear whether the rabbit C_κ genes are an exception or if many other examples of complex allotypes regulated by control genes will be found.

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TABLE I

TEST FOR THE PRESENCE OF LATENT ALLOTYPES IN SERA OF NORMAL RATS

Strain	Nominal RI-1 type	Number	Volume tested	Number inhibiting binding	% Inhibition (range)
Latent RI-1[a]					
WF	b	11	50 µl	10	12.9-36.7
LEW	b	5	50 µl	5	23.9-38.4
AVN	b	1	50 µl	1	23.5
BN	b	2	50 µl	2	34.3-42.9
Total		19		18	
Latent RI-1[b]					
ACI	a	12	50 µl	10	14.6-59.3
F344	a	2	50 µl	2	32.7-40.5
Total		14		12	

RIA as described in Materials and Methods.

TABLE II

ALLOTYPE SUPPRESSION IN F_1 RATS

Strain	RI-1 type	Rat No.	Age (weeks)	Allotype suppressed	Concentration of immunoglobulin (mg/ml) containing RI-1	
					a	b
(WFxF344)F_1	b/a					
		1	6	a	3.3×10^{-3}	4.32
		2	6	a	3.3×10^{-3}	4.45
		3	6	a	2.3×10^{-3}	2.92
(LEWxACI)F_1	b/a					
		1	6	a	0.4×10^{-3}	0.53
		2	6	a	0.9×10^{-3}	0.98
		3	6	a	0.5×10^{-3}	0.61
		2	16	a	11.5×10^{-3}	n.d.
		3	16	a	12.5×10^{-3}	n.d.
(ACIxLEW)F_1	a/b					
		1	6	b	0.91	$\leq$0.1
		2	6	b	1.29	$\leq$0.1
		3	6	b	0.63	$\leq$0.1
(F344xWF)F_1	a/b					
		1	6	n.d.	1.49	1.42
		2	6	n.d.	1.76	1.16
		3	6	n.d.	0.89	1.32

Neonatal rats were allotype-suppressed as described in Materials and Methods and their sera were individually tested for RI-1^a and RI-1^b by RIA at the age indicated. Fifteen µl of sera were used to measure the amount of RI-1^a immunoglobulin in rats suppressed for the RI-1^a specificity. In all other cases, 4×10^{-2} µl of sera were tested. n.d. = not done.

TABLE III

RI-1 TYPING OF WILD RATTUS NORVEGICUS

	RI-1 type		
Location	a	a/b	b
Los Angeles, CA	0	5 (5)	0
Seal Beach, CA	2 (2)	5 (4)	3 (3)
Philadelphia, PA	8 (2)	1 (1)	0
TOTAL	10 (4)	11 (10)	3 (3)

Rats were typed by inhibition of passive hemagglutination. Parentheses indicate the number confirmed by RIA.

TABLE IV

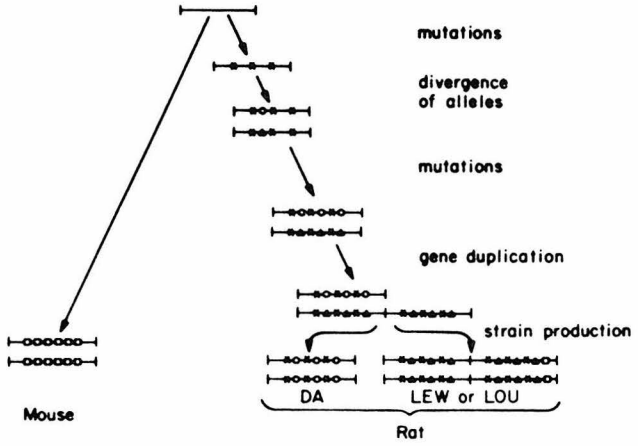
RI-1 TYPING OF WILD RODENTS

Inhibitor sera		Log_2 Hemagglutination titre		RIA-% Inhibition		
Strain or species	Location	ACIαWF + WF-SRBC	WFαACI + ACI-SRBC	ACIαWF + ^{125}I-WFIg	WFαACI + ^{125}I-ACIIg	RI-1 type
None		8	8	0.0	0.0	-
WF		0	8	95.4	5.8	b
ACI		8	0	1.9	105.7	a
Rattus rattus	Los Angeles, CA					
	1	0	8	100.5	5.6	b
	2	0	8	100.0	4.1	b
	3	0	8	105.2	9.7	b
	4	0	8	103.3	0.2	b
Neotoma fuscipes	Los Angeles, CA					
	1	8	7	15.3	3.2	-
	2	8	7	15.2	0.7	-
	3	8	7	2.6	-1.8	-
	4	7	7	15.1	-0.9	-
	5	7	7	10.8	n.d.	-

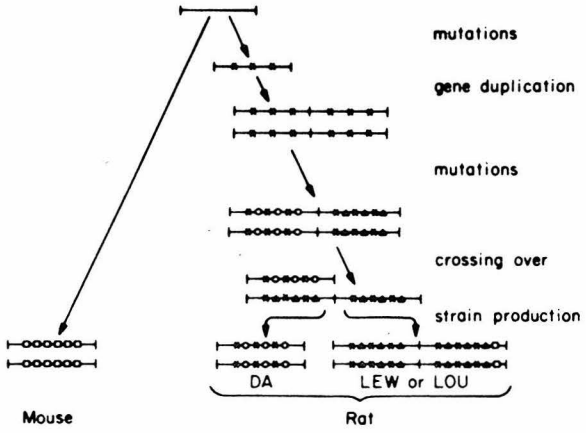
Rodents were tested for allotype by inhibition of passive hemagglutination and radioimmunoassay as described. For the RIA, 5 µl of competitor serum were tested.

Figure 1. Genetic models for the expression and evolution of the rat C_K allotypes. (a) Classical alleles. (b) Alleles by crossing-over. (c) Alleles by gene duplication and a control mechanism.

a. Classical Alleles



b. Alleles by Crossing Over



c. Alleles by Control Mechanism

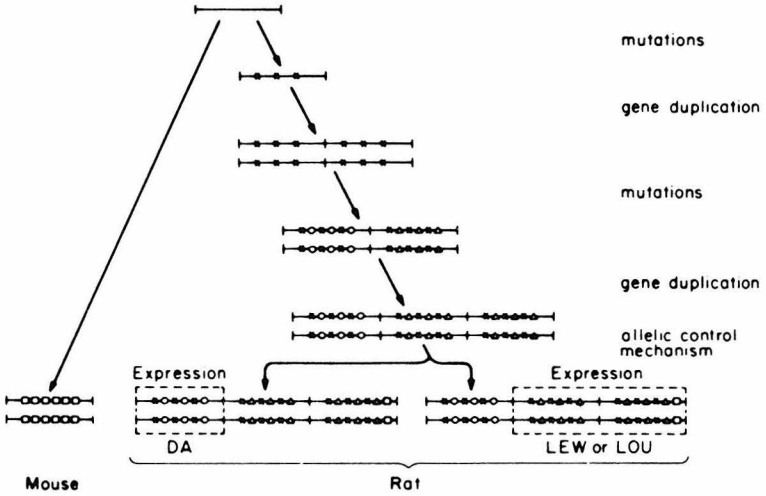


Figure 1

Figure 2. Standard curve for anti-allotype sera. Sepharose-coupled anti-allotype was mixed with various amounts of unlabeled immunoglobulin (Ig) for one hour before addition of labeled antigen. Percentage of inhibition of binding is plotted against concentration of unlabeled immunoglobulin. Slope and intercept of the lines determined by the method of least squares.

o; Sepharose-ACIαWF, ^{125}I-WF and WF Ig unlabeled competitor;

Δ; Sepharose-ACIαWF, ^{125}I-WF and ACI Ig unlabeled competitor;

□; Sepharose WFαACI, ^{125}I-ACI and ACI Ig unlabeled competitor;

x; Sepharose WFαACI, ^{125}I-ACI and WF Ig unlabeled competitor.

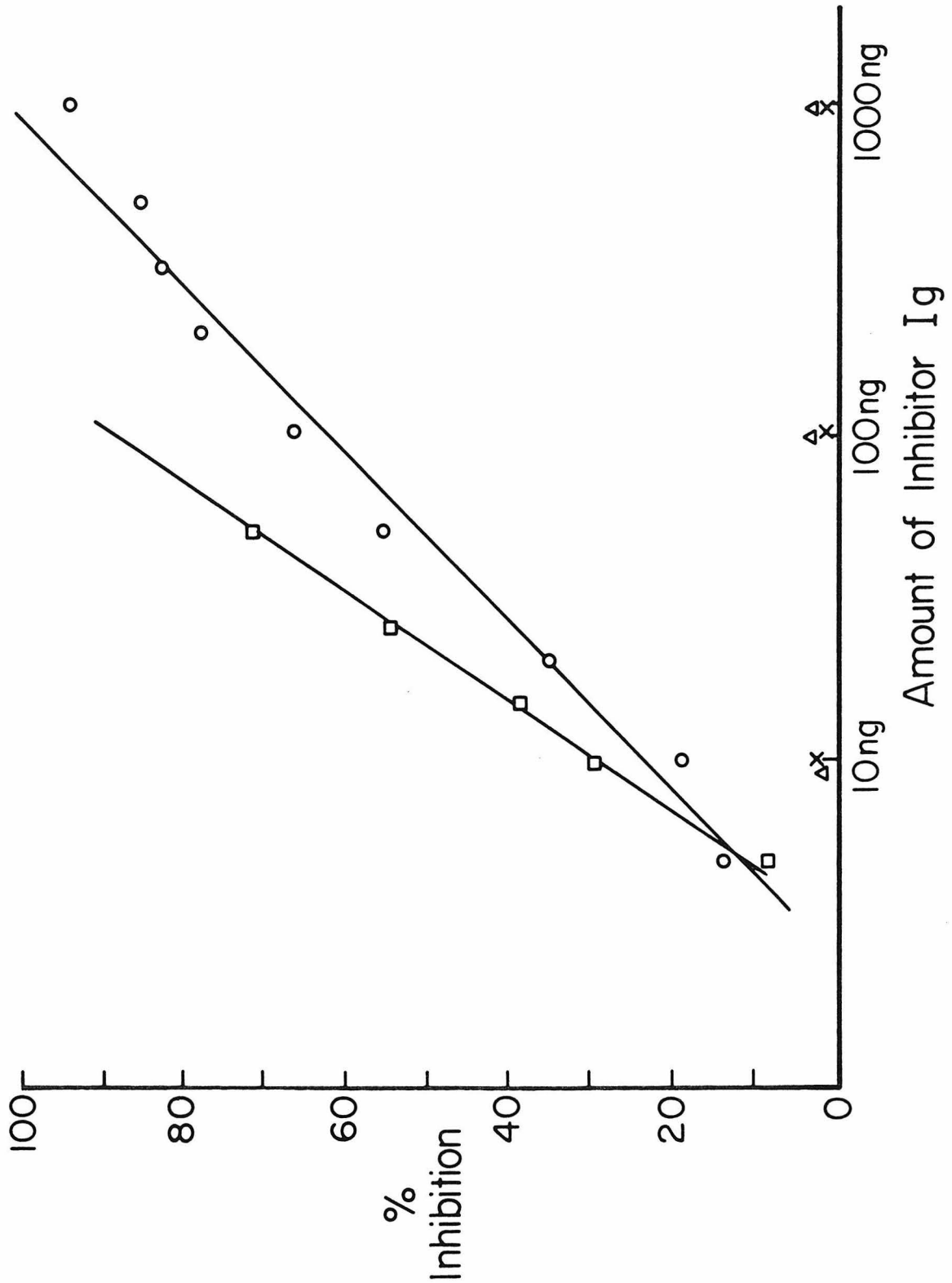

100
80
60
40
20
0
% Inhibition
10ng
100ng
1000ng
Amount of Inhibitor Ig

Figure 2

Figure 3. Sera from individual rats having material cross-reactive with unexpected allotypes was concentrated by pressure dialysis and then added to anti-allotype reagents to test for latent allotype as described. Volume of competitor serum plotted on the abscissa is the equivalent volume used had the serum not been concentrated. In all cases shown, RI-1^b rats were tested for RI-1^a expression.

(a) ●, WF rat (No. 11) serum. □ , LEW rat (No. 05) serum .

(b) o, AVN rat (No. 02) serum. □ , WF rat (No. 10) serum.

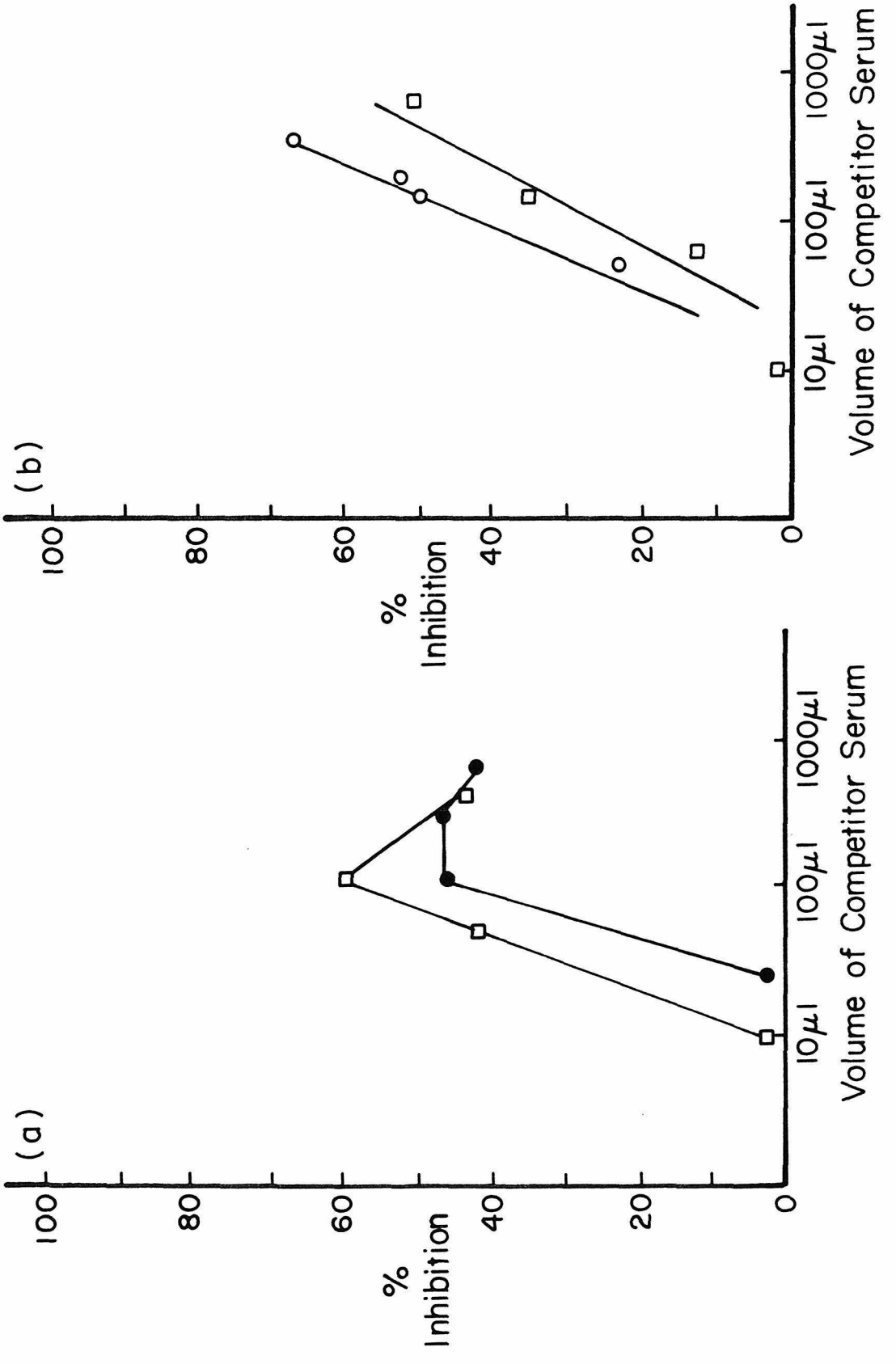

(a)
(b)
100
80
60
40
20
0
% Inhibition
10µl
100µl
1000µl
Volume of Competitor Serum

Figure 3

Chapter 2

SOME QUESTIONS AND CONTROVERSIES CONCERNING THE T-CELL ANTIGEN-BINDING RECEPTOR

B and T lymphocytes are unique in their capacity to respond specifically to antigens. While the range of the immune response can encompass diverse antigens, individual lymphocytes can recognize only a very few antigenic determinants. The set of determinants recognized by a cell result from the specificity of the antigen-binding receptor expressed on the surface of that lymphocyte. For B cells, it is known that the receptor molecule is an immunoglobulin.[1,2] For T lymphocytes, the structure of the receptor is completely uncharacterized. Although it has been a decade since the publication by Simonsen and his collaborators of the landmark paper entitled "The Elusive T Cell Receptor,"[3] the adjective "elusive" is often used to describe the antigen receptor today.

A. T Cell Development and Function. T lymphocytes arise from stem cells in the bone marrow and T-cell precursors migrate to the thymus where they become immunocompetent.[4] These virgin T cells leave the thymus and subsequently seed T-cell-specific domains of peripheral lymphoid organs.[4-6] Some of the different stages of this maturational pathway are characterized by differences in the expression of cell-surface alloantigens such as Thy-1, TL and Lyt 1, 2, and 3.[7]

T cells have at least two roles in the immune system. First, they regulate the immune response by providing signals that help or suppress other lymphocytes. Second, they are cytotoxic for cells that display antigenic determinants on their surface. The lymphocytes that carry out these roles can be divided into at least three separate functional subclasses: helper T cells,[8,9] suppressor T cells[10] and killer T cells.[11] T lymphocytes undergo an antigen-independent differentiation that results in commitment to one of these functional subclasses.[12,13] The presence of cell-surface alloantigens is correlated with the functional subclass; Lyt-1 positive cells tend to be helper cells,[9,12] Lyt 2,3 positive cells are cytotoxic or suppressor cells,[12,13] and I-J encoded molecules are expressed primarily on cells involved in suppression.[14]

Within the last five years, a number of techniques have become available that permit the isolation and propagation of functional cloned T lymphocytes. The two most commonly employed methods for immortalizing T cells are fusion to a drug-sensitive thymona cell line (usually BW5147)[15,16] or continued restimulation with antigen and/or T-cell growth factor (Interleukin-2). These cloned T cells have already been used in a large number of studies that have greatly expanded our knowledge of lymphocyte function. In addition, the specificity of these cloned cells proves that T lymphocytes do not passively acquire any part of their antigen-binding receptor from B lymphocytes.

B. T Cell Antigen Recognition: Results from Functional Studies. Experiments using molecules with precisely defined antigenic determinants, such as insulins,[19] cytochromes[20] and fibrino-peptides,[21] have demonstrated the ability of T lymphocytes to discriminate between very closely related antigens. The description of Ir gene defects (Section B.3) may indicate, however, that the range of T-cell immune responses is more restricted than antibody-antigen interactions. In addition, there is some controversy over whether T cell receptors must recognize a different type of antigenic determinant than immunoglobulins. In a number of cases, T and B cells responding to the same antigen recognize different antigenic determinants,[19,22] while the results of other experiments suggest the same determinant is probably recognized.[23-25] A major difference between B cell and T cell antigen recognition concerns the major histocompatibility complex (MHC). The MHC or H-2 complex of the mouse encodes at least two classes of cell-surface molecules (Figure 1).[26,27] The class I molecules include the transplantation antigens encoded by the H-2K, H-2D, H-2L and H-2R genes. Transplantation antigens are found on most of the cells in an organism. Class II molecules or Ia antigens, encoded by the H-2I region of the MHC, are present primarily on B lymphocytes and various types of macrophages. Studies of T-cell responses have indicated three ways in which the MHC influences antigen

recognition by T cells.

1. MHC Alloreactions. One to five percent of the T lymphocyte population can respond to stimulating cells from a mouse strain bearing a different set of H-2 alleles (H-2 haplotype).[28-30] By contrast, T cells responding to conventional antigen may be present at a frequency of $1/10^4$ to $1/10^5$.[31] Cytotoxic T cells responding to MHC differences tend to recognize class I gene products,[32] while cells which proliferate in response to MHC differences (predominantly helper T cells) tend to recognize class II molecules.[32,33] The extremely high frequency of MHC alloreactive cells has posed two interesting problems to immunologists. First, many different MHC haplotypes exist in any species, yet each different haplotype is recognized by many responding T cells. The high frequency responding to a single haplotype has therefore raised questions regarding the clonal selection theory and the notion that a single lymphocyte synthesizes just one antigen-receptor. Can we explain the high frequency by assuming that there is a great degree of cross-reactivity between different MHC alleles, or must the possibility of pluripotent responding T cells be seriously considered? Second, exposure to MHC alloantigens and the generation of a cellular immune response to these molecules occurs as a result of either organ transplantation in hospitals or laboratory experiments. Why is it that T cells are preoccupied with responding to antigens that are not an integral part of pathogenic organisms and may only rarely have served as immunogens throughout vertebrate evolution?

2. MHC Restriction. Many T lymphocytes do not recognize antigen alone, but respond to antigen in conjunction with products of their own MHC.[34] This requirement for MHC recognition is often referred to as MHC restriction and is illustrated in Table I for the case of cytotoxic T cells responding to virally infected cells. The specificity of the cytotoxic T cells is tested in a short-term (4 hour) assay. This assay allows the readout of the functional capability of primed cells generated by immunization, but requires too short a time to permit the generation of a new

immune response.[35] Thus, after immunization with virus, no response can be detected by A haplotype lymphocytes to the B haplotype target cells (line 2), even targets differ at the MHC and can therefore certainly be recognized by some A cells. The cytotoxic T cells generated are highly antigen-specific, since virus X does not cross-react with virus Y (line 3). The cytotoxic cells, however, will not kill any cell infected with virus X, but only those cells that share the MHC haplotype of the immunizing cells. A-X targets are killed but not B-X, although both cells display presumably identical viral-encoded polypeptides (lines 4,5). The surprising phenomena of MHC restriction have profoundly influenced concepts of T-cell antigen recognition. In addition, MHC-restricted antigen-recognition has led to new explanations for the polymorphism of MHC gene products,[36] the nearly ubiquitous tissue distribution of the transplantation antigens,[37] and the puzzling immune response (Ir) gene effects (Section B.3, 38). MHC restriction of antigen recognition is not a property solely of cytotoxic T cells. As with MHC alloreactive cells, there appears to be a division of labor such that Lyt-1 cells which provide help recognize antigen in conjunction with class II products, while Lyt-2,3 cells including cytotoxic T cells and some suppressors recognize antigen in the context of class I products. [39]

Originally it was proposed that MHC restriction occurred because T cells interacted with other lymphocytes or target cells via a "physiological" interaction of self-MHC with self-MHC.[40] Two kinds of experiments indicate that a like-like interaction of MHC-encoded molecules does not occur and that T cells recognize self-MHC using a set of receptors that bind these molecules. First, individual T lymphocytes express more MHC molecules than they are capable of recognizing as self. For example, cytotoxic T cells bearing a recombinant MHC haplotype (K^dD^s) express both K^d and D^s transplantation antigens but contain separate populations of effector cells that respond to antigen plus K^d or antigen plus D^s but not both.[41] Second, experiments utilizing bone marrow chimeras or thymic transplants have

indicated that the MHC molecules which can be recognized by MHC restricted T cells are not determined by the T lymphocyte genotype but result from a developmental process which may occur in the thymus.[42]

Two major kinds of theories have arisen to explain the apparent dual specificity of T lymphocytes (Figure 1). One theory proposes that an antigen-specific T cell has two receptors, one to bind the antigen and a second specific for self-MHC molecules.[37,43,44] The two binding sites could be on different polypeptide subunits on the same molecule or could even be on the same polypeptide, as long as these sites are separate. Two receptor theories maintain that the antigen-specific cells cannot be activated unless both receptor sites bind their respective ligands. Therefore, neither free antigen alone, nor self-MHC are sufficient to trigger a T-cell response.[43] The second class of theories proposes that the T cell has a single receptor or recognition site.[34,45] This site does not bind a self-MHC molecule or antigen alone, but it does bind a complex or neoantigenic determinant formed by the interaction of these two molecules. The complex of antigen and MHC molecule is sometimes referred to as "altered-self." This type of theory leads to rather simple explanations for a number of immune phenomena (such as the high frequency of MHC alloreactive T cells) but it must postulate a close interaction between MHC molecules and all types of antigens which is difficult to rationalize.

3. Ir Gene Effects. Immune response (Ir) genes that map to the I region of the MHC control the level of antibody production and T-cell proliferation to a number of different antigens.[26,46] Low responsiveness or Ir gene defects in the response to an antigen are inherited as recessive Mendelian traits. Low responsiveness is specific both for a particular Ir gene allele and a particular antigenic determinant. In addition to the Ir genes, polymorphic cell-surface molecules (Ia antigens or class II molecules) have been mapped to the I region.[46] Recent evidence has indicated that these Ia antigens are in fact responsible for the Ir gene defects.[47-49] A similar set of

phenomena which are controlled by genes encoding transplantation antigens has been described for cytotoxic T cells. Low responsiveness specific for certain H-2K or H-2D alleles in combination with certain viruses is well characterized.[38,39] While it can be argued that this is not strictly an Ir gene defect since the low responsiveness of cytotoxic T cells do not map to the I region, the effect of the class I gene products on cytotoxic T cell responses is completely analogous to the effect of class II products upon helper T cells.

The specificity of the Ir gene effect led to the proposal in 1972 that Ia antigens or the products of closely linked genes are T-cell antigen-binding receptors.[50,51] However, there appear to be a limited number of polypeptides encoded in the I region and it is therefore unlikely that Ia antigens themselves are the T-cell receptor.[52,53] Today it is widely believed that the Ir gene defects are a consequence of MHC-restricted antigen recognition by T lymphocytes.[27,46] Since MHC restriction itself is poorly understood, there is no general agreement as to the mechanism involved in the Ir gene defects. Most evidence supports the hypothesis that the defect results somehow from the inability of T cells to respond to a particular combination of antigen and MHC molecule.[54-56] This occurs because this combination (altered-self) closely resembles self[57] and is tolerated, or because of a number of hypothetical rules concerning the expression and somatic diversification of T-cell antigen receptors.[37,44] An alternative hypothesis (determinant selection) states that the Ir gene defect is not a problem of T-cell antigen recognition but resides instead in the inability of antigen presenting cells (B cells and macrophages) or target cells to form an MHC-antigen complex.[58]

In summary, functional studies of immune T cells have provided insight into the T-cell receptor problem. From the antigen-recognition viewpoint, there appear to be two major categories of T cells—those that react to differences in MHC alloantigens and those that react to conventional antigens in association with self-MHC antigens.

In addition, MHC-encoded molecules can influence the level of the immune response to antigens. These results have raised a number of interesting problems whose resolution depends upon a more detailed knowledge of the biochemistry of MHC-encoded molecules and T-cell antigen-binding receptors. These include: 1) What is the number and specificity of antigen receptors present on T lymphocytes? What is the number of polypeptides per receptor? 2) What differences are there between receptors expressed by different functional subclasses of T cells? Why is there an association between the T-cell functional subclass and the class of MHC molecule which must be recognized? Are there T-cell isotypes? 3) How do the receptors on MHC alloreactive cells differ from these on lymphocytes responding to conventional antigens? Why is there such a high frequency of these MHC alloreactive cells? 4) If each T lymphocyte has two antigen-binding sites, how does activation of the MHC alloreactive cells differ from the MHC-restricted activation of cells responding to conventional antigens? 5) What is the mechanism whereby MHC genes influence the immune response? Is a complex formed between antigen and self-MHC molecule? Can relevant structural differences between antigen receptors in low and high responder mice be defined? 6) How does the thymus influence the expression of antigen-receptors by developing T lymphocytes?

A number of theories have been developed which attempt to answer one or more of the above questions.[34,37,43-45,51,57-61] Some of the issues, particularly the number of antigen-binding receptors expressed by MHC-restricted T lymphocytes, have inspired a rather lively debate.[37,62,63] Most theories suffer from the inclusion of ad hoc assumptions that are not currently testable and they will therefore not be considered in detail. For example, several dual receptor theories assume, based on Jerne's proposal,[64] that the library of germline genes for T-cell antigen receptors encodes a set of polypeptides that recognize all the MHC alleles present in a given species.[37,44] Not only is it difficult to understand how the T-cell receptor and MHC

gene families can coevolve in this way, but the hypothesis is almost impossible to falsify. While, characterization of the genes encoding T-cell antigen-binding receptors is essential to understanding T lymphocyte specificity and MHC function, clearly, a large number of experiments, including functional and biochemical studies combined with in vitro mutagenesis of cloned MHC genes will be required to answer the lengthy list of questions we have posed.

C. Serology and Biochemistry of T-Cell Antigen-Binding Receptors. After the discovery that lymphocytes can be divided into B-cell and T-cell subsets, there was a consensus that immunoglobulin would constitute the antigen receptor for both types of lymphocytes.[3] However, solid evidence for the presence of immunoglobulin on the surface of T lymphocytes was difficult to obtain. When we began our experiments, there was no longer any consensus and it was believed that the antigen-binding receptor on T lymphocytes might consist of either: 1) conventional immunoglobulin including V and C regions of B cell light and heavy chains;[65,66] 2) portions of conventional immunoglobulin, usually V_H regions, presumably expressed along with unique T-cell constant regions;[67,68] 3) MHC-encoded molecules;[50,51] or d) molecules not encoded by any of the above gene families. Various combinations of these possibilities have also been suggested.[43,63,69-72]

In our analysis of the structure of T-cell antigen-binding receptors, we focused exclusively on the possibility that T lymphocytes express immunoglobulin genes. There were several reasons for doing this. First, a variety of cloned B cell immunoglobulin DNA probes had recently become available. The rearrangement of these genes and their patterns of transcription had been well characterized in B lymphocytes allowing us to unambiguously answer questions concerning immunoglobulin gene expression and rearrangement in T cells.[73] Second, a considerable amount of evidence was consistent with immunoglobulin gene expression by T lymphocytes (Section D). Finally, identification of nonimmunoglobulin genes which

might encode T-cell antigen-binding receptors was hindered by the fact that there existed no reliable assay for gene products involved in T-cell antigen recognition. This appears to be the case today (see Chapter 6).

D. Evidence for Immunoglobulin Gene Expression by T Lymphocytes. T cells do not generally react with conventional anti-mouse immunoglobulin or anti-constant region sera.[74,75] In fact, positive or negative selection of lymphocytes using these sera is a major method for the separation of B and T lymphocytes. However, expression of immunoglobulin constant regions in T cells remained controversial for many years.[76] There are numerous reports of anti-immunoglobulin sera reacting with T lymphocytes.[69,77] Some anti-κ chain sera effect T-cell function,[78] and there are reports that anti-μ,[66,79] anti-γ[66,80] and anti-κ sera[66] bind to the surface or precipitate proteins from T lymphocytes. How can we reconcile these findings with a much larger body of negative results? It is likely that the use of poorly characterized polyclonal antisera or the impurity of the cell populations tested could lead to false positives for immunoglobulin expression. However, it is also possible that T cells do synthesize immunoglobulin polypeptides but that these proteins may be masked on the cell membrane and/or present in very small amounts. Differences in antisera, T cell populations tested or the method of preparation of cell-surface proteins might then be critically important. The possibility that immunoglobulins are synthesized by T cells was further supported by the finding that κ, α and μ chain RNAs were detected in thymocytes and T lymphomas.[81-84] However, interpretation of these experiments is complicated by the fact that cloned immunoglobulin nucleic acid probes were not used.

While the evidence in favor of constant region expression is not convincing, a large body of work supports the idea that T cells utilize variable gene segments to encode part of their antigen-specific receptor.[23,24,43,85-87] It has been demonstrated that anti-idiotypic sera can effect T-cell functions <u>in vivo</u> and <u>in vitro</u>, bind to

T-cell antigen-binding material secreted or shed from the cells, and bind to the surface of T lymphocytes. Experiments indicate that the idiotype-positive protein is synthesized rather than passively acquired by the T cells and that the expression of idiotype is linked to genes coding for the heavy chain constant region. In the response to the hapten NP (4-hydroxy-3-nitrophenyl-acetate), monoclonal anti-idiotypic sera were shown to react with cloned T cells.[88] Thus, in this instance the expression of idiotype by T lymphocytes cannot be attributed to artifacts resulting from the heterogeneity of the cells tested or uncharacterized antibodies in polyclonal sera. Idiotype was often detected in the absence of serological determinants present on light or heavy chain constant regions.[67,68,85,86] Linkage of idiotype to the genes coding for heavy chain constant regions,[89] but not to V_κ or C_κ genes,[90,91] led to the hypothesis that the T-cell antigen-binding receptor contains heavy chain variable regions without light chain variable or heavy and light chain constant regions. Despite the years of intensive investigation, over 200 publications, and some very impressive experiments, the expression of the V_H gene segments by T lymphocytes has remained controversial. There are several reasons for this. First, although idiotype-positive T lymphocytes have been described in the responses to over a dozen different antigens, a number of critical experiments have never been successfully performed. For example, in very few instances has it been directly demonstrated that anti-idiotypic sera can inhibit binding of antigen to T lymphocytes.[70,92,93] In addition, these sera have never been successfully used to immunoprecipitate biosynthetically-labeled molecules from the surface of T lymphocytes. Second, although idiotypic determinants may be present on T-cell antigen-binding receptors, these experiments may have been overinterpreted since in principle one cannot infer close structural homology based upon serological cross-reactivity. This problem is discussed in Chapter 4.

In the following chapters we describe attempts to detect immunoglobulin gene

expression in T cells. Specifically, we asked three questions: 1) Do T lymphocytes rearrange or transcribe immunoglobulin J and C gene segments? 2) Do T lymphocytes responding to an antigen transcribe V_H gene segments homologous to those transcribed by B cells responding to the same antigen? 3) Do T cells utilize any V_H gene segments as part of their antigen receptor? By employing cloned immunoglobulin DNA probes and cloned T lymphocytes, we avoided some of the ambiguities that plagued experiments based upon detecting cross-reactive antigenic determinants. We demonstrated that T cells do not use J and C gene segments as part of their antigen-binding receptor. Neither do they transcribe V_H gene segments homologous to those transcribed by B cells responding to the same antigen. In addition, we were able to make a strong argument against T-cell expression of any V_H gene segments. However, the T-cell antigen receptor remains elusive.

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Table I

Immune T Cells Recognize Viral Antigen Plus MHC Gene Products

Responders[1] (cytotoxic T cells)	Stimulators (immunizing cells)	Targets	Cytotoxic response[4]
1. A lymphocytes[2]	A-X[3]	A	-
2. A lymphocytes	A-X	B	-
3. A lymphocytes	A-X	A-Y	-
4. A lymphocytes	A-X	A-X	+
5. A lymphocytes	A-X	B-X	-

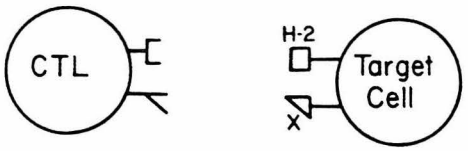
[1]Generally a secondary response is measured.[39] In a typical protocol, virus is first injected into an animal and after several days lymphoid tissue containing immune cells (responders) is removed. The immune lymphocytes are cultured for three to five days in the presence of virally-infected cells (stimulators). MHC restriction has also been observed for primary responses.[94] This table is adapted from Reference 45.

[2]A, B = different MHC haplotypes.

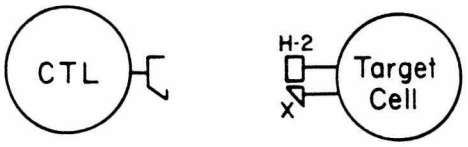
[3]X, Y = different viruses or polypeptides encoded by different viruses on the surface of infected cells.

[4]Target cells for the cytotoxic assay are labeled with ^{51}Cr. Lysis of the targets by cytotoxic T cells is measured by monitoring release of ^{51}Cr into the culture medium.[35]

H-2 RESTRICTION OF T-CELL REACTIONS



Dual Receptor Hypothesis



Altered-self Hypothesis

Figure 1

Chapter 3

HELPER AND KILLER T CELLS DO NOT EXPRESS B CELL IMMUNOGLOBULIN JOINING AND CONSTANT REGION GENE SEGMENTS

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HELPER AND KILLER T CELLS DO NOT EXPRESS B CELL IMMUNOGLOBULIN JOINING AND CONSTANT REGION GENE SEGMENTS*

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The immune system is characterized by both the specificity and breadth of its response. The generation of an immune response depends upon the specific recognition of antigens by lymphocytes. There are two classes of lymphocytes: B cells capable of secreting large quantities of immunoglobulin and T cells that can carry out facilitation or help of lymphocyte responses (1, 2), suppression of lymphocyte responses (3), or cytotoxicity directed against appropriate target cells (4). A T cell is apparently committed to a single functional subclass prior to any exposure to antigen (5, 6).

The antigen-binding receptor on the surface of B lymphocytes is an immunoglobulin molecule composed of two identical light chain and two identical heavy chain polypeptides. Both light and heavy chains have variable regions responsible for antigen binding and constant regions responsible for various effector functions such as complement fixation. The immunoglobulins are encoded by three unlinked gene families: two light chain families, κ and λ, and a heavy chain family. In mice, there are one or more constant region genes for each light chain family, and at least eight heavy chain constant region genes (μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, α, and ϵ). Each mature B cell and its progeny can respond to only one or a few antigenic determinants because it can synthesize only one light chain and one heavy chain variable region. However, during their development, B cells switch from the expression of IgM molecules containing μ heavy chains to the expression of other immunoglobulin classes containing different heavy chain constant regions, while continuing to express the same V_H region and light chain (7, 8).

Recent experiments employing recombinant DNA techniques have clarified some of the molecular events necessary for B-cell antibody expression. Both the commitment to express single V_H and V_L regions, and the switch in expression of heavy chain constant region are characterized by DNA rearrangements. In the mouse, the light chain is encoded by three separate gene segments—V, J[1] (joining), and C (constant) (9). The V and J gene segments together code for the variable region. For mouse κ chains, there are multiple germline V_κ gene segments ($\geq$200) (10) and four J_κ gene segments (11, 12). The heavy chain is encoded by four gene segments—V_H, D (diversity), J_H and C_H, and in some cases an M (membrane) exon (13–16). The V

* Supported by grant AI 10781 from the U. S. Public Health Service.

[1] *Abbreviations used in this paper:* C, constant; C_T, constant region; D, diversity; J, joining; J_T, J gene segment; kb, kilobase; V, variable.

gene segment (and presumably the D gene segment) expressed in a given B cell is rearranged from its germ-line context and becomes contiguous with a J gene segment that is located a few kilobases 5′ from a C gene (11–13, 17). DNA sequences between the V and J gene segments are deleted during this rearrangement (11, 18, 19). Heavy chain constant-region switching takes place through replacement of the μ constant-region gene and some of its flanking sequence by another C_H gene (20).[2]

Individual T cells can also respond specifically to one or a few antigenic determinants (21–23). However, the molecular properties of the T-cell antigen-binding receptor are a subject of great controversy (24). Most attempts to define the receptor have relied on antisera made to either B-cell derived immunoglobulin (25–31) or to responding T cells (32, 33) in conjunction with immunological or genetic experiments. There is considerable evidence that T cells synthesize V_H regions without light chains or any of the C_H regions expressed in B cells (29–31), but there are a number of reports of the expression of light chains and C_H regions in T cells (25–28, 34, 35).

The development of two relatively new techniques has persuaded us to reexamine the controversy regarding immunoglobulin synthesis by T cells. First, cell lines grown in T-cell growth factor or Interleukin II (36) provided us with large numbers of mature, functional T cells that are entirely free of contaminating B cells and have a well-defined antigen specificity. Second, using the methods of recombinant DNA research, we have obtained cloned DNA probes for immunoglobulin heavy and light chain J and C gene segments. We have used our DNA probes to ask whether these J and C gene segments are rearranged and transcribed into RNA by T cells. Because both the T cells and the DNA probes are homogeneous and well characterized, we hoped to avoid the ambiguities of some of the earlier experiments concerning T-cell expression of immunoglobulin constant regions. In addition, by using probes for the heavy and light chain J gene segments, we could obtain information on T-cell expression of the 3′ part of the variable region. We have obtained convincing data that the B-cell immunoglobulin light and heavy chain J and C gene segments are neither rearranged nor expressed in the monoclonal helper and killer T cells that we have analyzed.

Materials and Methods

T Cells. BALB/c Cum mice were originally obtained from Cumberland View Farms, Clinton, Tenn., and have since then been bred in our animal colony at the California Institute of Technology. C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Thymus tissue was dissected from 2- to 4-wk-old BALB/c mice killed by inhalation of ether. Mice were injected with 0.1 ml of India ink prior to sacrifice to facilitate visualization and removal of parathymic lymph nodes. The antigen specificity and cell-surface phenotype of our monoclonal T cells are presented in Table I. WEHI-22 is an irradiation-induced BALB/c T lymphoma (27). It has been reported to synthesize large amounts of a 68,000 mol wt protein that cross-reacts with an antiserum made against mouse immunoglobulin (28). WEHI-22 cells were a gift of Dr. Noel Warner, University of New Mexico, Albuquerque, N. Mex. and were grown in suspension culture in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum. The helper cell line HT-1 (23) and the alloreactive killer cell line CTLLi6 (37) were grown in RPMI-1640 medium (Grand Island Biological Co.) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

[2] Davis, M. M., S. Kim, and L. Hood. DNA sequences mediating heavy chain switching in alpha immunoglobulin genes. Manuscript submitted for publication.

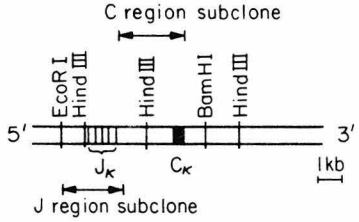
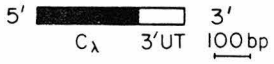
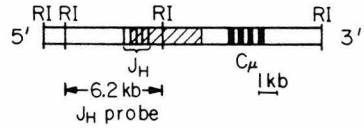
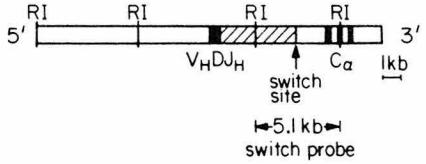
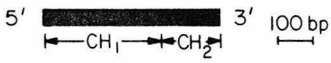
TABLE I

Monoclonal T Cells Analyzed with Cloned Immunoglobulin DNA Probes

Cell line	Strain of origin	Function	Antigen specificity	Cell surface phenotype	Description	Reference
HT-1	C57B1/6J	Helper	Sheep erythrocytes Helps H-2^b B cells	θ^+, Lyt-1$^+$	Growth factor-dependent cell line	23
CTLLi6	C57B1/6J	Killer	H-2^d	θ^+, Lyt-2$^+$	Growth factor-dependent cell line	37
WEHI-22	BALB/c	?	?	θ^+	T lymphoma	27

TABLE II

Cloned Immunoglobulin Probes

Region	Source		Reference
C_κ (C region subclone)	Subclone from BALB/c genomic DNA		38
J_κ (J region subclone)	Subclone from BALB/c genomic DNA		38
C_γ (pAbγ_1-7)	cDNA from HOPC 2020 mRNA		A. Bothwell. Personal communication.
J_H	Fragment from BALB/c genomic DNA		20; this report
Switch (p5.1)	Subclone from BALB/c myeloma DNA		20
C_μ (p104Eμ6)	cDNA from M104E mRNA		14
C_α	cDNA from S107 mRNA		20

A supernatant fraction from concanavalin A-stimulated lymphocytes enriched for Interleukin II was prepared as described (23) and added to the medium at a concentration of 10 U/ml.

Immunoglobulin Probes. Description of the immunoglobulin DNA probes employed in these studies is presented in Table II. The C_κ and J_κ gene segments were subcloned from a liver DNA

genomic clone. These subclones were a gift of Dr. Michael Steinmetz, California Institute of Technology, Calif. (38). A C_λ cDNA clone, pAbλ_1-7, was a gift of Dr. Alfred Bothwell and Dr. David Baltimore, Massachusetts Institute of Technology, Boston, Mass. A probe containing the J_H gene segments was prepared by electroeluting a 6.2 kilobase (kb) EcoRI fragment from the ChSp μ27 clone originally isolated as a C_μ-containing clone from a collection or library of BALB/c sperm DNA clones (20). The probe containing heavy chain constant region switch sites was subcloned from the Ch603α6 clone. This clone was originally isolated from a library made from the DNA of the IgA-producing myeloma McPC603 (17). The original α6 clone contains the 603 V_H gene segment joined to a J_H gene segment, with C_α gene found 6.8 kb to the 3′ side of the J_H gene segment. The subclone is a 5.1 kb EcoRI fragment containing intervening DNA sequence and the 5′ half of the C_α gene (17). In the embryo, 1.5 kb of this intervening sequence is found adjacent to the C_μ gene and not the C_α gene (20). The point at which C_μ and C_α adjacent sequences are joined is denoted the switch site. Five examples of C_H switching have thus far been analyzed, including three from rearranged genes of C_α-producing myeloma tumors (17, 20),[2] one from a $C_{\gamma 1}$ producer (39, 40), and one from a $C_{\gamma 2b}$ producer (41). All the active genes from these myelomas have C_μ flanking sequence extending from a J_H gene segment towards the expressed C_γ or C_α gene. The point of deletion of the C_μ flanking sequence or switch site is not the same for every tumor, but they are all found within about 300 base pairs of one another. The 5.1 kb subclone we used as a probe should hybridize with restriction fragments containing all these known switch sites as well as several kb of surrounding DNA.

Preparation of DNA and Southern Blots. High molecular weight DNA was prepared according to the method of Blin and Stafford (42). Embryo DNA was prepared from whole 12-d BALB/c embryos. DNA digested with restriction enzymes was electrophoresed in 0.7% agarose gels, transferred to nitrocellulose filters (43), and hybridized with nick-translated probes (44). Unless otherwise noted, hybridizations were performed at 68°C in 1.0 M NaCl/0.045 M Trisodium citrate/0.2% bovine serum albumin/0.2% Ficoll/0.2% polyvinylpyrrolidone/0.1% $NaDodSO_4$/100 μg/ml denatured Salmon sperm DNA/25 μg/ml poly-rA. Subsequent to hybridization, filters were washed extensively in 0.30 M NaCl/0.03 M Trisodium citrate/0.1% $NaDodSO_4$/0.1% sodium pyrophosphate at 68°C and autoradiographed.

Preparation of RNA and Northern Blots. Purified immunoglobulin mRNA containing the α (MOPC167) and μ (M104E) heavy chain constant regions and the κ (S107) light chain constant region were prepared as described (17). Total RNA was prepared from T cells homogenized in guanidinium thiocyanate and centrifuged through cesium chloride (45). RNA preparations electrophoresed on agarose gels and stained with ethidium bromide were undegraded, free of contaminating DNA, and had a 260 nm/280 nm absorbance ratio of about 2.1:1. ^{3}H-labeled RNA from sea urachin (*Strongylocentrotus purpuratus*) embryos (a gift of Dr. Frank Costantini, California Institute of Technology) was added at the beginning of the T cell RNA preparation as a recovery marker. Between 43 and 64% of the added counts per minute were present in the final RNA solution. From the absorbance of the mouse T cell RNA at 260 nm, and the yield of labeled *S. purpuratus* RNA estimated from recovered counts per minute, we calculated that WEHI-22 has 12.7 pg of RNA/cell, HT-1 has 5.8 pg/cell, CTLLi6 has 3.7 pg/cell, and thymus has 1.6 pg/cell. Polyadenylated [poly(A)$^+$] RNA was selected by oligo(dT)-cellulose chromatography (46). Poly(A)$^+$ RNA was denatured and electrophoresed on agarose gels containing 2.2 M formaldehyde (47). Denatured RNA was transferred directly onto nitrocellulose filters (B. Seed and D. Goldberg. Manuscript in preparation.), and filters were hybridized at 42°C in 50% formamide (48). In control experiments, ^{32}P-labeled RNA from Sindbis virus-infected cells (a gift of Charles Rice, California Institute of Technology) was electrophoresed on agarose gels, transferred to nitrocellulose filters, and incubated in hybridization and wash solutions. Virla RNA as large as 13.0 kb was efficiently transferred to filters. About 45% of the RNA remained bound to the nitrocellulose as measured by densitometry of viral RNA bands present on autoradiographs of the filter exposed before and after incubation in hybridization buffers. In one experiment, diazophenylthioether paper (a gift of Brian Seed, California Institute of Technology) rather than nitrocellulose, was used as a solid support to bind RNA.

Results

Experimental Strategy. T cell DNA was analyzed for rearrangement using the Southern blot technique (43). High molecular weight genomic DNA was digested

with a restriction enzyme and the resulting fragments were separated according to molecular weight in an agarose gel. The double-stranded DNA was then denatured so that single strands were available for hybridization. The size-separated and denatured DNA was transferred from the agarose gel to a solid support, generally a nitrocellulose filter, and the filter-bound DNA was hybridized with a radioactive-cloned DNA probe. The filter was autoradiographed, and restriction fragments, having sequences complementary to the probe, appeared as bands on the film. By comparing the band pattern in T cells with the band pattern obtained from germ-line DNA, we assessed whether a particular DNA sequence was rearranged. In analyzing the DNA from WEHI-22 and BALB/c thymus, cells from 12-d BALB/c embryos and adult livers provided a source of germ-line DNA. For the HT-1 and CTLLi6 cell lines, which come from C57BL/6J mice, DNA undifferentiated with respect to immunoglobulin genes was obtained from the cells of adult liver and kidney.

We used Northern blots to detect immunoglobulin sequences in T cell RNA. The experimental strategy is very similar to that employed to detect DNA rearrangements. Poly(A)$^+$ total cell RNA from T cells was size-separated on agarose gels, transferred to nitrocellulose filters, hybridized with radioactive immunoglobulin DNA probes, and the filters were then autoradiographed.

T Cell DNA Analyzed for Gene Rearrangement Using Light Chain Probes. The results obtained from hybridizing light chain DNA probes to restriction fragments of T cell DNA are presented in Table III and Fig. 1. All the bands present in the T cell DNA are also present in the germ-line DNA from the same strain, indicating that C_κ, J_κ, and C_λ sequences are not rearranged in the T cells. In a few cases, however, C57BL/

TABLE III

Sizes of Restriction Fragments Containing Light Chain Immunoglobulin Genes in T Cell and Nonlymphoid DNA

Digests	Probes								
	C_κ			J_κ			C_λ		
	Bam HI	Eco RI	Hind III	Bam HI	Eco RI	Hind III	Bam HI	Eco RI	Hind III
C57B16 DNA									
HT-1	13.0	15.0	4.3, 2.7	13.0	15.0	2.7	7.3	9.0	8.4, 3.2
CTLLi6	13.0	15.0	4.3, 2.7	13.0	15.0	2.7	7.3	9.0	8.4, 3.2
Liver or kidney	13.0	15.0	4.3, 3.5 2.7	13.0	15.0	3.5, 2.7	7.3	9.0, 6.6	8.4, 3.2
BALB/c DNA									
WEHI-22	13.0	15.0	4.3, 2.7	ND‡	15.0	ND	ND	9.0	ND
Thymus	13.0	15.0	4.3, 2.7	ND	15.0	2.7	ND	9.0	ND
Liver or embryo	13.0	15.0	4.3, 2.7	ND	15.0	2.7	ND	9.0	ND

* Sizes of restriction fragments containing immunoglobulin genes were estimated from the migration of molecular weight standards that included Eco RI- and Hind III-digested λ bacteriophage DNA, and pBr322 plasmid digested with both Ava I and Hind II. In every case, T cell DNA was electrophoresed in a lane adjacent to one of the nonlymphoid DNA samples. WEHI-22 and BALB/c thymus DNA were electrophoresed on the same gels; HT-1 and CTLLi6 DNA were electrophoresed on separate gels.

‡ ND, not determined.

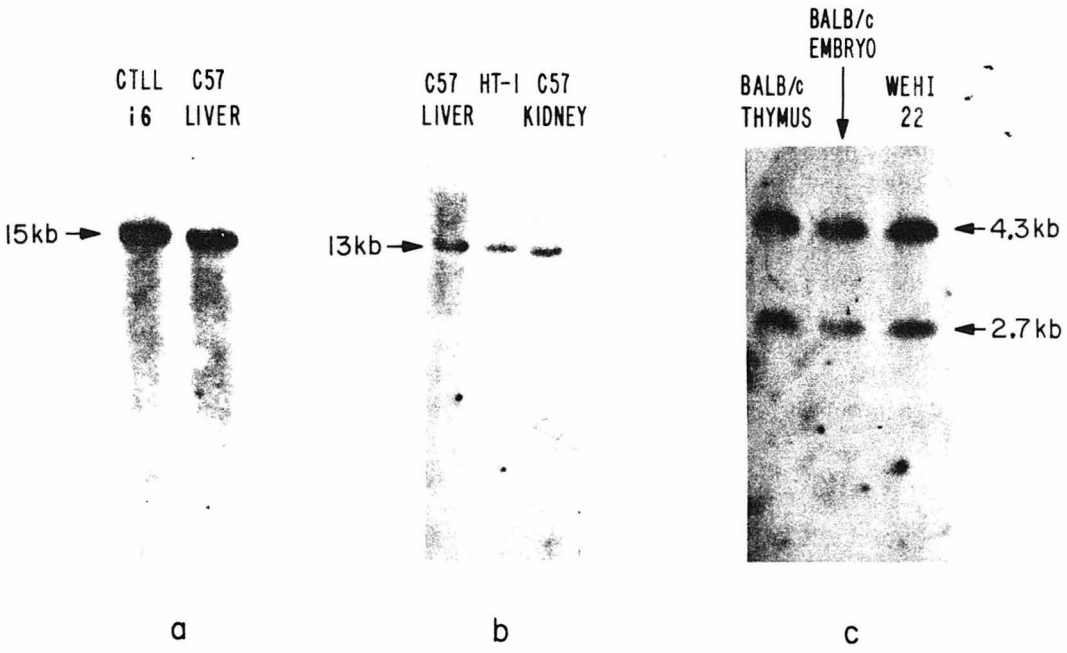


FIG. 1. T cell and nonlymphoid DNA hybridized with a radioactive C_κ probe. (a–c) are autoradiographs of DNA from separate gels. In (a) DNA samples were digested with restriction enzyme Eco RI; in (b) they were digested with Bam HI; and (c) they were digested with Hind III. The sizes of the hybridizing fragments are indicated. For experimental details see Materials and Methods.

6J liver and kidney DNA had a restriction fragment hybridizing with the light chain probes that was not apparent in the two monoclonal T cell lines (Table III). The extra band in C57BL/6J liver and kidney is sometimes faint, and a technical artifact probably accounts for this result. A number of much less likely explanations are formally possible, however, including a genetic polymorphism in C57BL/6J mice, a T-cell specific sequence deletion (as opposed to rearrangement), or even an immunoglobulin gene rearrangement specific to liver and kidney cells.

T Cell DNA Analyzed for Gene Rearrangement Using Heavy Chain Probes. The results obtained from hybridizing heavy chain probes to restriction fragments of T-cell DNA are presented in Table IV and Figs. 2 and 3.

We observed no rearrangement of J_H gene segments in the T cells tested. The 6.2 kb J_H probe contains a sequence at its 5′ end that is repeated in the mouse genome (P. W. Early. Unpublished observations.). To reduce the background caused by hybridization with these repeated sequences, filters hybridized with the 6.2 kb J_H probe were washed extensively in low salt conditions (20 mM cation at 68°C), that favor melting of imperfectly matched hybrid molecules. After the low salt washes, one (EcoRI and Hind III digests) or two (Bam HI digest) dark bands remained on the filter, and the sizes of these major bands are presented in Table IV. The Eco RI fragment from BALB/c DNA that hybridizes with the probe is the same size as the EcoRI fragment used to isolate the probe from a sperm DNA clone, suggesting that this major band is not simply a large tandem array of repeated sequences or some other artifact. A 5.1 kb subclone containing sequences involved in B-cell heavy chain

TABLE IV

Sizes of Restriction Fragments Containing Heavy Chain Immunoglobulin Genes in T Cell and Nonlymphoid DNA

Digests	Probes											
	J_H			Switch			C_μ membrane			C_α		
	Bam HI	Eco RI	Hind III	Bam HI	Eco RI	Hind III	Bam HI	Eco RI	Hind III	Bam HI	Eco RI	Hind III
C57B16 DNA												
HT-1	11.0	6.2	2.0	23.0	15.0	6.2	11.0	15.0	2.65	22.5	10.0	5.7
	1.0		0.9	14.0	10.0	5.7			1.25			
CTLLi6	11.0	6.2	2.0	23.0	15.0	6.2	11.0	15.0	2.65	22.5	10.0	5.7
	1.0		0.9	14.0	10.0	5.7			1.25			
Liver or kidney	11.0	6.2	2.0	23.0	15.0	6.2	11.0	15.0	2.65	22.5	10.0	5.7
	1.0		0.9	14.0	10.0	5.7			1.25			
BALB/c DNA												
WEHI-22	10.0	6.2	2.0	ND*	12.5	ND	11.0	12.5	2.65	22.5	9.5	3.7
	1.0		0.9		9.5, 4.8				1.25			
Thymus	10.0	6.2	2.0	ND	12.5	ND	11.0	12.5	2.65	22.5	9.5	3.7
	1.0		0.9		9.5, 4.8				1.25			
Liver or embryo	10.0	6.2	2.0	ND	12.5	ND	11.0	12.5	2.65	22.5	9.5	3.7
	1.0		0.9		9.5, 4.8				1.25			

For details see the legend for Table III.

* ND, not determined.

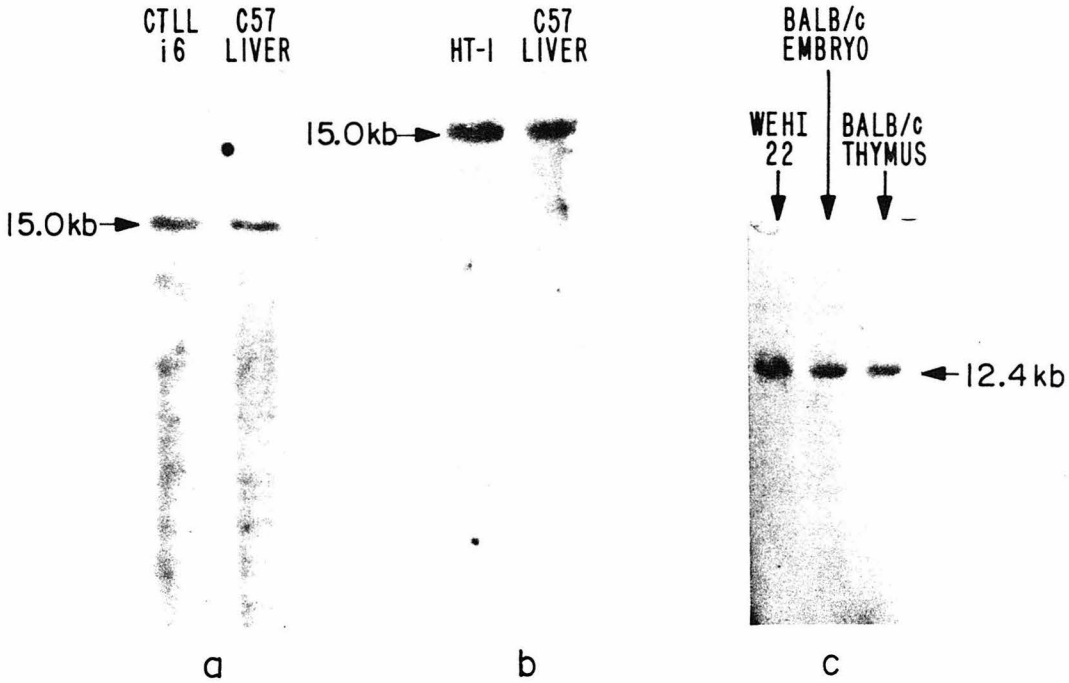


FIG. 2. T cell and nonlymphoid DNA hybridized with a radioactive C_μ probe. All DNA samples were digested with Eco RI. See Fig. 1 legend and Materials and Methods for details.

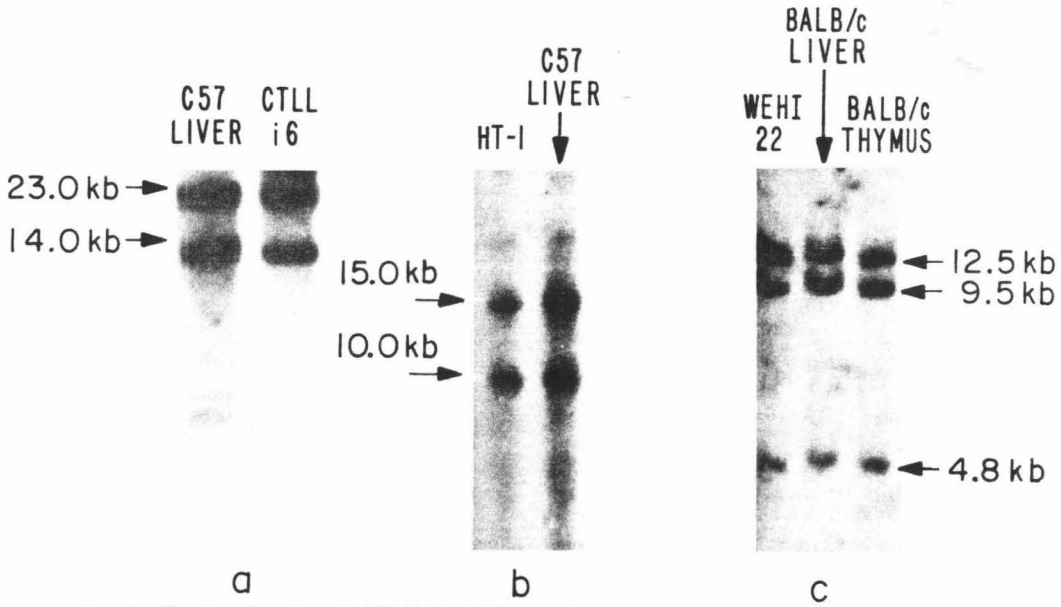


FIG. 3. T cell and nonlymphoid DNA hybridized with a radioactive probe containing heavy chain constant region switch sites. In 3a, DNA was digested with Bam HI, whereas in Figs. 3b and c the DNA was digested with Eco RI. See Fig. 1 legend and Materials and Methods for details.

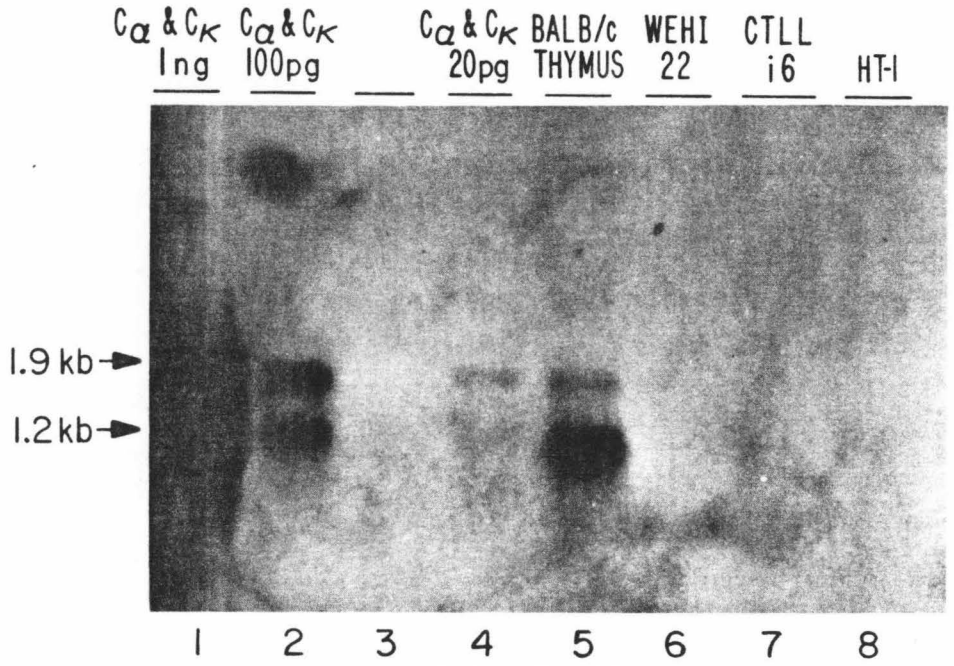


FIG. 4. Filter-bound poly(A)$^+$ RNA hybridized with C_κ and C_α probes. RNA molecular weight standards included *Escherichia coli* 16, and 23S ribosomal RNA, and murine 18 and 28S ribosomal RNA. Purified myeloma RNA is present in lanes 1, 2, and 4. When the probes are hybridized to separate filters, the C_κ RNA sequence in thymus is 1.2 kb, whereas the larger C_α RNA is 1.9 kb. We could detect 1.4 copies/cell of κ RNA and <1.0 copy/cell of α RNA on this autoradiograph.

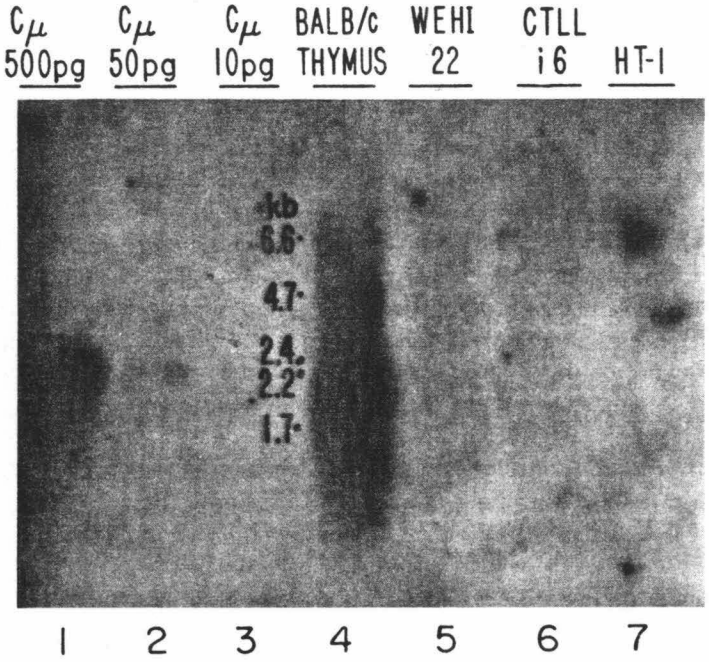


FIG. 5. Filter-bound poly(A)$^+$ RNA hybridized with a C_μ probe. RNA molecular weight standards included 26S and 42S Sindbis virus RNA and *E. coli*, and murine ribosomal RNA. Purified myeloma RNA standards are present in lanes 1, 2, and 3. Molecular weights of thymus RNA bands are indicated. We could detect 1.8 copies/cell or less of μ RNA on this autoradiograph. Detection limit varied with different samples because of different amounts of poly(A)$^+$ RNA (1–10 μg) added to the gel.

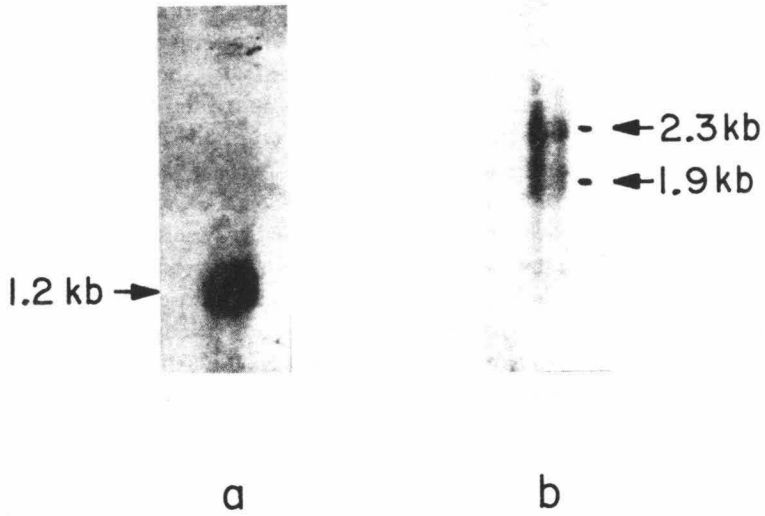


FIG. 6. Poly(A)$^+$ thymus RNA hybridized to C_κ and C_μ probes. (a) Poly(A)$^+$ thymus RNA was size-separated on an agarose gel and transferred to diazotized paper. RNA covalently bound to the diazotized paper was hybridized with a radioactive C_κ probe, and the paper was autoradiographed. (b) The paper with bound thymus RNA, as in (a), was washed in 95% formamide at 68°C to remove the radioactive C_κ probe. It was then hybridized with a C_μ probe and autoradiographed.

constant region switching was hybridized to the T cell DNA. No rearrangement of these sequences was observed (Fig. 2). The C_μ probe we used is a cDNA clone made from RNA coding for the membrane form of μ heavy chain. We have detected no rearrangement of the C_μ gene in the T cells (Fig. 3). The C_α gene was also present in the germline or undifferentiated configuration in all the T cells analyzed.

In addition, because of reports that T cells make a polypeptide serologically cross-reactive with the μ chain (25, 26), we hybridized the C_μ probe at 55°C to BALB/c and C57B1/6J liver DNA cut with various restriction enzymes. The filter was subsequently washed at 55°C. All other conditions were as described in Materials and Methods. These low temperature conditions should permit hybridization of two nucleotide sequences that are only 70% homologous (see Discussion). No bands besides those known to contain the C_μ gene hybridized with the probe, therefore the gene for the putative C_μ homologue was not detected in this experiment.

Northern Blot Analysis of T Cell RNA. Although gene rearrangement is closely associated with immunoglobulin gene expression in B cells, it is possible that T cells could express immunoglobulin genes without rearranging them. We therefore analyzed T-cell RNA for the presence of immunoglobulin sequences. Size-separated poly(A)$^+$ RNA from T cells was hybridized with ^{32}P-labeled C_κ (Fig. 4), C_α (Fig. 4), C_μ (Fig. 5), and C_λ (data not shown) probes. Purified immunoglobulin RNA were run on the same gels, and provided us with an estimate of our detection limit, which ranged from 10 to 50 pg of RNA in a series of five gels. Given the detection limit, the amount of poly(A)$^+$ RNA/cell (which is about 1% of the total cell RNA calculated in Materials and Methods) and the micrograms of RNA added per gel lane, we estimate that we could detect less than two molecules of immunoglobulin RNA/cell in every case. No immunoglobulin RNA was detected in three monoclonal T-cell lines. Whole thymus, however, contained ~150 pg of C_κ RNA (7 copies/cell), 30 pg of C_α RNA (0.7 copies/cell), and 680 pg of C_μ RNA (16 copies/cell) as judged by densitometric comparison of thymus RNA bands to the bands obtained with purified immunoglobulin RNA. In addition, a 4.7 kb species that hybridized with the C_μ probe was present on some gels at ~0.4 copies/cell. Whole thymus was not tested for the presence of C_λ RNA. The C_κ and C_α RNA in the thymus were found in the poly(A)$^+$ fraction only (data not shown), and were approximately the same molecular weight as the polysomal C_κ and C_α mRNAs found in B lymphocytes. The C_μ RNA was found in a diffuse band ranging from ~2.3 to 1.7 kb in size. The μ mRNA species in B cells are 2.7 and 2.4 kb (13). The relatively low molecular weight for C_μ sequences in thymus was observed in two separate RNA preparations, and a control experiment, in which both the κ and μ probes were hybridized to the same RNA, verified that degradation was not responsible for the diffuse band and decreased size. The thymus RNA sequences hybridizing with the κ probe appeared to be a single species of about 1.2 kb (Fig. 6a), whereas the sequences hybridizing with the C_μ probe had a characteristic broad molecular weight distribution, ranging in this case from ~2.6 to 1.7 kb, with major bands visible at ~2.3 and 1.9 kb (Fig. 6b).

Discussion

After 10 years of intensive investigation, the structure of the T-cell antigen receptor remains controversial. Approaches to T-cell antigen recognition molecules based on the use of antisera to immunoglobulins have failed to yield definitive results. In this

study we have employed cloned probes containing immunoglobulin coding and flanking genomic DNA sequences to test for rearrangement and expression of immunoglobulin genes in T cells. The experiments depend upon the ability of our labeled probes to hybridize with genomic DNA or poly(A)$^+$ RNA containing immunoglobulin sequences. This approach has two distinct advantages: (*a*) The physical and chemical basis of nucleic acid hybridization is well understood and we can estimate the degree of sequence homology required for hybridization. (*b*) We can detect events, such as the synthesis of RNA molecules, which occur at two copies/cell or less. Most of our experiments were performed on three cloned cell lines, eliminating the possibility of any B cell contamination of our DNA and RNA preparations. In addition, HT-1 and CTLLi6 are bona fide mature T cells with a defined antigen specificity and functional subclass.

There is No Rearrangement of Immunoglobulin J and C Gene Segments in the Monoclonal T Cells. We used the Southern blot technique to determine whether T cells rearrange immunoglobulin J and C gene segments. Such rearrangements are clearly involved in V gene expression and heavy chain constant region switching in B cells. A rearranged DNA sequence could, however, give a band pattern identical to embryo DNA on Southern blots for two reasons: (*a*) The sequence is fortuitously rearranged to a new restriction fragment the same size as the embryonic fragment. (*b*) The restriction enzyme employed cuts a fragment that does not span the part of the DNA sequence rearranged. For example, the restriction enzyme might cut a fragment from the middle of a C_H gene stretching several kb to the 3′ side of this gene. This cleavage would not detect DNA rearrangement in B cells because when the V and J gene segments are joined in these cells, all the sequences 3′ to the constant region remain in the germ-line configuration. To reduce the possibility of missing a rearrangement, we generally hybridized each of our probes to separate T cell DNA samples cut with three different restriction enzymes. This is particularly important for the C57BL/6J-derived T cells because the position of restriction enzyme sites with respect to the immunoglobulin genes is not as well characterized in this inbred strain as it is in BALB/c mice. In total, we have carried out 45 separate comparisons of the T-cell DNA with liver, kidney, and embryo DNA using the heavy chain probes, and another 27 with the light chain probes (Table III, Table IV, M. Kronenberg. Unpublished observations.). All the restriction fragments that hybridize with immunoglobulin probes in T cells also are present in the nonlymphoid DNA. The J_κ, C_κ, C_λ, J_H, C_μ, and C_α gene segments, as well as the defined heavy chain constant region switch sites, are therefore not rearranged in the T cells analyzed. Although we have not directly tested for the rearrangement of the C_δ or C_γ heavy chain genes, it seems unlikely that T cells express these sequences because the J_H gene segments and heavy chain constant region switch sequences are not rearranged. Because the J_λ gene segment should be on the same EcoRI restriction fragment as the C_λ gene in BALB/c, we can also infer that the J_λ gene segment is not rearranged in WEHI-22.

It is difficult to draw definitive conclusions from Southern blots of thymus DNA. If some thymus cells rearrange immunoglobulin genes, we would expect each clone of cells to generate a new restriction fragment when it joins a J gene segment to a particular V gene segment. The detection limit on our Southern blots was between 0.1 and 0.5 copies/cell. The concentration in thymus DNA of any rearranged fragment particular to a given clone of cells would therefore be too low to detect on Southern

blots. Blots on thymus DNA then can only rule out a rearrangement common to most thymus cells that might, for example, be involved in the inactivation of the immunoglobulin gene loci. There is no evidence for joining of variable gene segments to J_H and J_κ gene segments in thymus, and our data clearly indicate that monoclonal, mature T cells, which certainly developed in the thymus, do not rearrange these gene segments.

Monoclonal T Cells Do Not Synthesize RNA Containing Immunoglobulin Sequences. In addition to testing for gene rearrangement, we analyzed T cells for transcription of immunoglobulin genes. We could not detect any C_κ, C_λ, C_μ, or C_α RNA in the monoclonal T cells. Because of the susceptibility of RNA molecules to enzymatic degradation, conclusions based upon negative data from Nothern blots must be viewed with caution. However, poly(A)$^+$ T-cell RNA electrophoresed on agarose gels and stained with ethidium bromide appeared intact as judged by general size distribution and the staining of some residual 18 and 28S ribosomal RNA bands. In addition, spleen and S117 myeloma RNA, prepared and handled exactly as the T-cell RNA were, and run in parallel on the same gels, gave strong hybridization with the radioactive immunoglobulin probes (data not shown). Finally, Southern blot analysis has indicated that the C_κ, C_λ, C_μ, and C_α gene segments are found in the germ-line configuration. Thus, if we did miss a small amount of RNA synthesis, the transcribed constant region gene was not close to a V gene segment, and this transcription is therefore unlikely to be involved in antigen-receptor biosynthesis. It has recently been reported that WEHI-22 cells synthesize about three copies of C_μ RNA/cell (49). We retested our poly(A)$^+$ RNA from WEHI-22, under conditions where we should have been able to detect ~0.4 copies/cell, and found no hybridization with the C_μ probe (M. Kronenberg. Unpublished observation.). This discrepancy is most probably caused by some heterogeneity in the WEHI-22 cell line.

Thymocytes May Synthesize Some RNA with C_μ Sequences. In preparations of total cell RNA from thymus, we find small amounts of C_κ, C_μ, and C_α RNA in the poly(A)$^+$ fraction. We have no information as to whether the immunoglobulin RNA is in the nucleus, free in the cytoplasm, or on polysomes, nor is it entirely clear which cell type synthesizes the RNA.

The sizes of the κ and α RNA are similar to that found in antibody-secreting B cells. It is therefore unlikely that this RNA originated from cells with unrearranged κ and α genes. In our experiments, B-cell contamination may be a likely explanation for RNA containing κ and α sequences. Given the amount of immunoglobulin RNA in plasmacytomas (50), if our thymus preparations contained ~0.01% plasma cells, we would have obtained the C_κ and C_α hybridization that was observed on Northern blots. Storb et al. have previously reported the presence of C_κ and C_α RNA in thymocytes, although they detected about 50-fold more RNA/cell than we did (51, 52). Conflicting data have been obtained on the question of whether or not the C_κ RNA is synthesized by contaminating B cells (53, 54).

The unusual molecular weight distribution observed for C_μ RNA in the thymus is similar to that previously observed for thymocytes (53), and for 7 out of 13 different T lymphomas ([49]; and D. Kemp. Personal communication.). This suggests that the C_μ RNA that we have detected may originate from thymocytes rather than contaminating B cells. Because most thymocytes are not immunologically competent (55), one could speculate that immature T cells synthesize μ heavy chain before switching

to expression of T cell constant regions. T cell-derived μ RNA is transcribed from unrearranged DNA and does not contain either V_H or J_H gene segments (D. Kemp. Personal communication.). The relationship of this C_μ sequence transcription to eventual expression of antigen receptors that presumably contain V regions is unclear.

The Gene for a Putative T Cell Constant Region Must Have Diverged by at Least 30% from the C_μ Gene Segment. There are reports in the literature that T cells synthesize a polypeptide serologically cross-reactive with μ heavy chain (25, 26). In DNA cleaved with several restriction enzymes, we have found only one band containing a single gene (15) that hybridizes with the C_μ probe. We can obtain a rough estimate of how far a homologous C_μ gene must have diverged in order to have not cross-hybridized with the C_μ probe. Hybridization of a C_μ probe to a homologous gene will depend upon a number of conditions including salt, temperature, percent guanine and cytosine content of the DNA sequence, and the length of the hybridizing fragments (56). Under our standard hybridization conditions, we are ~30°C below the temperature at which 50% of hybrid molecules formed will separate (T_m) for perfectly matched sequences. We therefore estimate that a gene 25% divergent in DNA sequence from a probe can be detected, and this has been empirically verified (S. Crews. Unpublished observations.). For a 55°C hybridization, we might hope to detect a gene that is another 10% (i.e., 35%) divergent from our C_μ probe. Although there are uncertainties in the estimates, given our Southern blot results, if there is a polypeptide homologous to μ made by T cells, it is likely that the gene coding for this protein diverged by at least 30% from the B cell C_μ gene sequence.

The T Cell Receptor Genes May Have J and C Gene Segments that are Distinct from Those of Their B Cell Counterparts. In Fig. 7 is given our model of the organization of the genes encoding the B and T cell antigen receptors. Evolutionary considerations indicate that multigene families can duplicate to generate new families that can acquire different functions and interact with gene products of the old family (57). This suggests that the gene families encoding the B and T cell antigen receptors could have evolved from a common ancestral gene family. If so, they should share common or homologous gene elements and mechanisms of DNA rearrangement for gene expression. This has been demonstrated for the B-cell κ, λ, and heavy chain gene families (9, 11–13, 57). Our model is based upon these evolutionary considerations, data drawn from the literature indicating that B and T cells express the same V_H gene segments (29–33), and the data presented in this paper demonstrating that T cells do not express B-cell J and C gene segments. Several points should be emphasized. (*a*) We presume that T cells express V_H gene segments through a mechanism similar to the mechanism that has been defined for B cells. This implies that T cells express V_H gene segments in conjunction with T cell-specific constant region (C_T) genes, and that there are multiple C_T genes that are expressed differentially on the functional subclasses of T cells. We believe that the rearrangement of a V_H gene segment, with

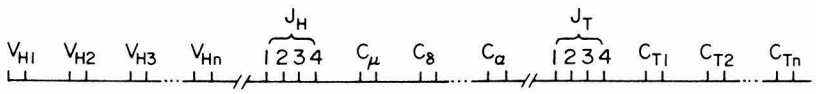


FIG. 7. A model of the genes encoding the B and T cell antigen binding receptors. Exons and intervening DNA sequences are not drawn to scale. Subscript T denotes gene segments expressed in T cells only. The position of the postulated J_T and C_T gene segment cluster with respect to the other indicated gene segments is unknown.

or without a D segment, to a T cell J gene segment (J_T) will place that V_H gene segment in the proper context for expression along with C_T genes. (*b*) The J_H gene segment codes for much of the third hypervariable region of B-cell derived antibody molecules (13). If T cells employ different J_H gene segments along with the B-cell V_H gene segments, then we might expect to find some differences in the antigen-binding specificities of T cells. This may partially explain experiments indicating that T cells recognize different antigenic determinants than B cells (58). In addition, some idiotypic markers that depend upon specific residues in the J region should be absent from T cell antigen binding receptors. This has implications for experiments employing anti-idiotypic reagents as probes for antigen-binding receptors and for the proposed regulation of immune responses via idiotypic antiidiotypic interactions. (*c*) Although there is much evidence for V_H expression, there is little evidence for V_L gene segment expression by idiotype-positive T cells (31, 32, 59). If T cells do express V_L gene segments, then the statements we make concerning the heavy chain gene family can be readily extended to include the light chain family. (*d*) If there is a C_T gene product that cross-reacts serologically with the μ chain, we have obtained data indicating the gene for this protein is not likely to be >70% homologous to the C_μ heavy chain gene sequence. This is not surprising because C_T genes probably diverged from B-cell C_H genes about the time of appearance of vertebrates with circulating immunoglobulin, and we would therefore expect only limited sequence homology between the B and T cell C gene clusters.

Summary

We have analyzed four kinds of T cells for rearrangement and expression of immunoglobulin genes. These cells include: (*a*) whole thymus; (*b*) WEHI-22, a T-cell lymphoma; (*c*) HT-1, an major histocompatability complex-restricted T helper line; and (*d*) CTLLi6, an H-2 alloreactive killer cell line. None of the B-cell joining and constant gene segments are rearranged in the T cells. The monoclonal cells do not express any C_κ, C_λ, C_μ, or C_α RNA species. Small amounts of C_κ, C_α, and C_μ sequences are present in RNA prepared from the thymus, although the significance of this RNA for T-cell antigen receptor synthesis is uncertain. The data support the hypothesis that expression of B-cell joining and C gene segments is unnecessary for T-cell helper and T-cell killer activity.

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Chapter 4

GAT-SPECIFIC T AND B CELLS DO NOT TRANSCRIBE SIMILAR HEAVY CHAIN VARIABLE REGION GENE SEGMENTS

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GAT-SPECIFIC T AND B CELLS DO NOT TRANSCRIBE SIMILAR HEAVY CHAIN VARIABLE REGION GENE SEGMENTS

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Running title: The GAT T cell receptor gene is not Ig V_H GAT

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The generation of a humoral immune response depends on the interaction between B lymphocytes, which synthesize immunoglobulin, and T lymphocytes, which regulate the B cell response. Although an organism can respond to a large set of diverse antigens, individual T and B cells synthesize receptors which can recognize only a few related antigenic determinants. B cells bind antigen through cell-surface immunoglobulin, a molecule composed of two identical heavy (H) and two identical light (L) polypeptide chains. During differentiation, individual B cells undergo a series of DNA rearrangements, becoming committed to the synthesis of immunoglobulin of a single specificity. The heavy chain variable region is composed of three separate gene segments, V_H, D, and J_H, which become joined to form the expressed heavy chain gene (1). Similarly, the light chain variable region is encoded by two distinct gene segments, V_L and J_L, which become joined by a DNA rearrangement to produce the transcriptionally active light chain gene (2).

T cells also exhibit a high degree of antigen specificity, but the molecular nature of the T cell receptor for antigen is not well characterized. Previous work has shown that the T cell antigen-binding receptor is not a conventional immunoglobulin; many functional murine T cells do not transcribe the C_H, C_L, J_H, or J_L gene segments which are required for encoding a complete immunoglobulin molecule (3). However, serological studies have suggested that T cells involved in the responses to synthetic polypeptides (4, 5), carbohydrate residues (6), alloantigens (7), haptens (8, 9), and protein antigens (12) express idiotypic determinants characteristic of immunoglobulins generated in response to the same antigens. Anti-idiotypic sera have been used to affect T cell function both in vivo and in vitro (6, 9, 13, 14) and are capable of binding antigen-specific factors secreted by T cells (4, 5, 8, 9). Furthermore, in a number of systems, the expression of idiotypic determinants by T cells is linked to the V_H or C_H, but not the C_κ, locus (9, 15-19). Two conclusions have often been drawn from these experiments. First, it has been proposed that T

cells transcribe V_H gene segments joined not to the immunoglobulin D, J_H and C_H gene segments, but to T cell-specific constant region segments (10). Second, it has been asserted that T and B cells use the V_H gene repertoire in a similar fashion such that both types of lymphocyte might transcribe similar V_H gene segments in response to the same antigen (11).

We set out to determine whether T and B cells which respond to the same antigen and share idiotypic determinants do in fact transcribe the same V_H gene segments. We have chosen to analyze the murine immune response to the synthetic polypeptide, glutamic acid^{60}, alanine^{30} and tyrosine^{10} (GAT), for two reasons. First, the B cell response to GAT displays limited diversity, as evidenced by a restricted isoelectric focusing pattern (20), and the presence of a predominant idiotype, CGAT (21). In addition, the sequences of five out of five heavy chains derived from immunoglobulins that bind GAT and four out of five heavy chains derived from proteins binding the related synthetic polymer GA are highly similar, even though only four of the antibodies express the CGAT idiotype (22). Therefore it is likely that there is one or a few highly homologous V_H gene segments expressed in the murine B cell response to GAT. Second, GAT-reactive T cells of different functional classes have been cloned and many display CGAT idiotypic determinants (5, 23, 24). Therefore, we can address directly whether a set of GAT-specific T cell clones, some of which are idiotype-positive, transcribe the GAT V_H gene segment.

We isolated a cDNA clone encoding the GAT V_H from a GAT-reactive B-cell hybridoma. Using the GAT V_H probe, we tested RNA from nine GAT-specific T-suppressor hybridomas, one GAT-specific helper hybridoma, and two GAT-specific T helper cell lines. Although six of these T cells express the CGAT idiotype on secreted, antigen-binding factors, we were unable to detect a GAT V_H transcript in any of the T cell RNAs. We therefore conclude that T and B cells responding to the same antigen do not use the same V_H gene segment. The large body of data

suggesting the expression of idiotypic determinants on T cells must be reinterpreted in light of this result.

Materials and Methods

Cells. The GAT-specific B cell hybridoma, F9-238.9, was produced by the fusion of GAT-primed DBA/2 spleen cells and the HAT-sensitive myeloma cell line, P3-X63-Ag8 (25). The resulting hybridoma synthesizes both the MOPC21 (γ, κ) immunoglobulin derived from the myeloma parent and a GAT-binding immunoglobulin (μ, κ) (Fig. 1). The F9-238.9 cells were kindly provided by Dr. Ronald N. Germain (NIH, Bethesda, MD).

The suppressor T-cell hybridomas were derived by fusion of splenic T cells from mice immunized with either GAT, GT, T_SF1 plus GT, or GAT conjugated to macrophages and BW5147, a HAT-sensitive T lymphoma of AKR origin (23, 24). The helper T-cell hybridoma resulted from a fusion between cultured T cell blasts derived from lymph node cells reactive with GAT and BW5147. The T_H lines were derived from lymph node lymphocytes of mice primed in vivo with soluble GAT and were maintained in vitro on GAT plus supernatant from ConA stimulated rat spleen cells. The characteristics of the T cell clones that we analyzed are summarized in Table I. Before RNA isolation, the T-cell hybridomas were expanded in liquid culture from frozen stocks and assayed for GAT-specific reactivity by previously published methods (23, 24, 26). The cells were harvested and the resulting pellets frozen at -70°C.

RNA Isolation. The frozen cell pellets were lysed by vortexing in 4 M guanidinium thiocyanate (Tridom, Hauppage, NY), 25 mM sodium acetate, pH 5, 0.5% sodium sarkosyl, 0.33% antifoam (Sigma, St. Louis, MO), 1 M β-mercaptoethanol. The RNA was purified by centrifugation through a cushion of cesium chloride (27), and the poly(A)-containing fraction enriched by two passages over oligo(dT) cellulose (28) (Type 3, Collaborative Research, Waltham, MA). The RNA concentration was

determined spectrophotometrically.

Construction of cDNA Library. A cDNA library was constructed using 15 μg poly(A)-RNA from F9-238.9 GAT-specific B cells. Synthesis of the first cDNA strand by AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL) was carried out with minor modifications of published procedures (29-32). The synthesis was initiated at random sites along the mRNA by the addition of sheared calf thymus DNA primer (33). The primer was prepared by digesting calf thymus DNA with DNase I to an average size of 10-15 nucleotides. RNasin (Biotec, Madison, WI) was added to the reverse transcriptase reaction to inhibit degradation of the RNA template (34).

The alkaline hydrolysis of the RNA and subsequent synthesis of the second cDNA strand by E. coli DNA polymerase I (Boehringer Mannheim, Indianapolis, IN) were performed as previously described (29, 30, 35). Following digestion of the single-stranded loop with Aspergillus S1 nuclease (Sigma, St. Louis, MO) (29, 30), the double-stranded cDNA was fractionated by gel filtration on a Biogel A150 (BioRad, Richmond, CA) column. The cDNA greater than 500 nucleotides in length, with an average size of approximately 800 bases, was tailed at each 3' end with 10-15 dCTP nucleotides using terminal deoxynucleotidyl transferase (New England Nuclear, Boston, MA) (36).

The cDNA was then annealed to the tetracycline-resistant plasmid, pBR322, which had been digested with the restriction enzyme PstI (New England Biolabs, Beverly, MA) and tailed at the resulting 3' ends with 8-10 nucleotides of dGTP. The annealed DNA was used to transform E. coli strain MC1061 (37), as described (38) and the transformants were selected by plating on nitrocellulose filters (Millipore, Bedford, MA) placed on media containing 15 μg/ml tetracycline. Replica filters were prepared for hybridization or frozen for storage as described (39).

Isolation of pGAT50, the GAT V_H cDNA Clone. The cDNA library was screened with a mixture of 32 pentadecamers (5'CAT$^{A}_{G}$TA$^{A}_{T}_{G}_{C}$GT$^{A}_{G}$TC$^{T}_{C}$TT3'), synthesized at Hoffmann LaRoche (Nutley, NJ) and kindly provided by Dr. Ken Wieder. The probe mixture was labeled at the 5' end with T4 polynucleotide kinase and [γ-^{32}P]dATP (ICN, Irvine, CA) (40). Prior to screening with the radioactive probe, plasmid DNA was amplified for 36 h on plates containing 12.5 μg/ml chloramphenicol. The bacterial cells then were lysed and prepared for hybridization with the probe as described (39). Duplicate filters were pre-hybridized at 23° for 3 h in hybridization buffer, consisting of 5X SET (0.75 M sodium chloride/0.15 M Tris, pH 8, 5 mM ethylenediaminetetraacetic acid), 5X Denhardts solution (41), 10 μg/ml each of poly (rA), poly (rC), and poly (rG), 0.5% sodium dodecyl sulfate, and 250 μg/ml denatured, sheared salmon sperm DNA. The probe was added to the filters in fresh hybridization buffer at 0.5 pmol/ml or 5.5 x 10^6 cpm/ml. Following 16 h of hybridization at 23°, the filters were washed in 5X SET, 0.1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate at 23° and exposed to Kodak XAR-5 film with an intensifying screen at -80°.

DNA Sequencing. DNA from the GAT V_H clone pGAT50 was digested either with PstI or PvuII (New England Biolabs, Inc., Beverly, MA), subcloned into the bacteriophage vector, M13mp8 (Bethesda Research Laboratories, Gaithersburg, MD) and sequenced by the dideoxy method (42). Also, pGAT50 cleaved with StuI (New England Biolabs, Beverly, MA) and labeled at the 5' ends with T4 polynucleotide kinase was digested with BglI and PvuI (New England Biolabs, Beverly, MA) and the labeled fragments purified and sequenced by the Maxam-Gilbert technique (43).

Northern Blots. Poly(A)-containing RNA was denatured, electrophoresed in agarose gels containing formaldehyde (44) and blotted to nitrocellulose (45). E. coli 16S and 23S rRNAs and murine 18S and 28S rRNAs were electrophoresed in parallel as

molecular weight standards. The filters were hybridized at 42° in formamide and dextran sulphate (Pharmacia, Piscataway, NJ) as previously described (45), with the addition of 5 μg/ml liver poly(A)-minus RNA. The blots were washed in 0.3 M sodium chloride, 0.03 M sodium citrate, and 0.1% sodium dodecyl sulfate, pH 7. at 50° and then exposed to Kodak XAR-5 film using an intensifying screen.

Hybridization probes. The following cloned DNAs were labeled to a specific activity of 2-8 x 10^8 cpm/μg by nick-translation (46, 47). 1. C_μ probe. Plasmid pSpμA1 (48) was digested with four restriction endonucleases, BamHI, KpnI, EcoRI and XbaI, to produce a restriction fragment containing the exons encoding $C_\mu 3$, $C_\mu 4$ and half of $C_\mu 2$. This restriction fragment was purified by electroelution from a preparative agarose gel. 2. MOPC 21 probe. A cDNA contianing virtually the entire MOPC21 heavy chain variable region gene was cloned into pBR322 (49). 3. V11 probe. A 3.5 kb SauIIIA restriction fragment, containing the genomic V11 gene segment (50), was subcloned into pBR322. V11 is a member of the TEPC15 gene family. DNA was kindly provided by Dr. Johanna A. Griffin (University of Alabama, Birmingham, AL). 4. V14A probe. A BamHI-EcoRI restriction fragment containing most of the genomic V_H gene segment, V14A, was subcloned into pBR322 (Stephen Crews, unpublished). The V14A sequence is very similar to the gene encoding the J606 myeloma heavy chain. The DNA was given to us by Dr. Stephen Crews (California Institute of Technology, Pasadena, CA). 5. S107 probe. A cDNA clone containing the V_H gene segment expressed by the S107 myeloma was previously isolated (2). S107 is a member of the TEPC15 gene family. The DNA was prepared by Dr. J. A. Griffin. 6. GAT50 probe. The derivation of the pGAT50, the GAT V_H cDNA clone is described in this manuscript. 7. H-2 class I probe. pH-2IIa was subcloned from a cDNA encoding a class I gene of the d haplotype (51). The DNA was provided by Dr. Michael Steinmetz (Basel Institute, Switzerland). 8. J_H probe. A 2 kb fragment containing all four J_H gene segments was excised from the plasmid

pSpμA1 (48) by digestion with HpaII (New England Biolabs, Beverly, MA) and purified as described above. 9. $\underline{C_\kappa \text{ probe}}$. A 6 kb BamHI-HindIII fragment containing the germline C_κ gene was subcloned in pBR322 from a genomic clone. The DNA was prepared by Dr. M. Steinmetz (Basel Institute for Immunology, Basel Switzerland).

Results

Isolation of pGAT50, a GAT V_H cDNA Clone. To isolate a cDNA clone containing the GAT immunoglobulin V_H coding region, we had to overcome two technical problems. First, the μ RNA is long, approximately 2.4 kb in length and the V_H region is located at its 5' end. Conventionally, cDNA synthesis is initiated by an oligo(dT) primer hybridized to the poly(A) tail found at the 3' end of most eukaryotic messenger RNAs. Therefore, to obtain a clone containing the entire GAT V_H would require synthesis of a cDNA greater than 2 kb in length. Since cDNAs this long are rare, we initiated synthesis using short fragments of calf thymus DNA as a primer. This primer could anneal at various points along the mRNA and the cDNA synthesized would derive from the 5' end of the RNA more frequently than with an oligo(dT) primer. Therefore, the probability of cloning the V_H sequence should be increased.

The second technical problem arose because the GAT B-hybridoma, F9-238.9, contains approximately 10 times more γ heavy chain mRNA derived from the myeloma parent than μ mRNA containing the GAT V_H. This is demonstrated by hybridizing a blot containing the B cell RNA with a germline J_H probe capable of detecting all heavy chain messenger RNAs. Hybridization to the 1.9 kb MOPC21 γ mRNA species is significantly more intense than hybridization to the 2.4 kb GAT heavy chain μ mRNA (Fig. 1). In addition, the F9-238.9 cells secrete approximately one tenth as much μ as γ heavy chain (Kraig, unpublished observation). To distinguish between the clones containing the GAT V_H and the more prevalent clones encoding the MOPC21 V_H, initially we screened the cDNA library with a C_μ probe. Although several clones containing some V_H sequence were isolated, no cDNA clones

containing the entire GAT V_H gene were identified. Since the NH_2-terminal amino acid sequence of several GAT-binding immunoglobulin heavy chains had been determined, it was possible to synthesize an oligonucleotide probe, complementary to the RNA sequence predicted to encode amino acid residues 31-35 (Fig. 2). Because there exist ambiguous positions in the reverse translation from amino acid into nucleotide sequence, the complements of all 32 possible coding sequences were synthesized. We hybridized the pool of labeled pentadecamers to the cDNA library under conditions which would distinguish between MOPC21 V_H, which has no more than 12 of 15 nucleotides identical to any of the 32 pentadecamers, and the GAT V_H, which should be identical to one of the probes at all 15 positions. Of 6000 cDNA clones screened, one, designated pGAT50, hybridized to the pool of radioactive pentadecamers and was characterized further.

The nucleotide sequence of pGAT50 and the sequencing strategy used are summarized in Fig. 3. The cDNA clone contains 106 nucleotides from the 5' untranslated (UT) region, a sequence capable of encoding a hydrophobic 19 amino acid leader peptide, complete V_H, D, and J_H gene segments and 72 nucleotides of the C_μ gene. The V_H, D, and J_H gene segments have rearranged in a continuous translational reading frame, indicating that the cDNA clone encodes a functional V region. The amino acid sequence predicted from pGAT50 (Fig. 3) agrees with the 33 residues of published NH_2-terminal protein sequence data for the F9-238.9 heavy chain (22). The D is identical to $D_{FL16.1}$ (52) and the J gene segment used is J_H2 (53). The clone also contains 72 nucleotides whose sequence is identical to that published for the NH_2-terminal portion of the first domain of C_μ (54).

GAT-specific T Cells Do Not Transcribe the GAT V_H Sequence. RNA was extracted from 10 different GAT-specific T-cell hybridomas and two T-cell lines (Table I) and the poly(A)-containing RNA purified by two or three sequential passages over oligo(dT)-cellulose. Ten μg of each T hybridoma RNA were separated according

to molecular weight on a denaturing gel, blotted to nitrocellulose, and hybridized with the radioactive pGAT50 probe. There was no evidence of a T-cell mRNA species which hybridized to the GAT V_H probe (Fig. 4). Similarly, there was no GAT V_H sequence in 3-6 μg of poly(A) RNA from two T_H lines, BB02' and BD01' (data not shown). The GAT V_H sequence could, however, be detected when as little as 50 ng of poly(A)-containing RNA from the B-cell hybridoma were loaded on the gel (Fig. 4).

The T cell RNA was shown to be present and undegraded by a positive control hybridization using pH2-IIa, a cDNA encoding an $H\text{-}2^a$ class I polypeptide (Fig. 5). All of the T cell RNAs contained an mRNA approximately 1.9 kb in length which hybridized to the H-2 probe and encodes the H-2 class I polypeptide localized in the cell membrane. In addition, several of the T_S hybridoma RNAs had a second mRNA of about 1.8 kb which hybridized to the class I probe. This smaller species of mRNA had been observed previously in liver and possibly encodes a secreted H-2 class I polypeptide (55).

It is possible that V_H sequences encoding T cell-derived GAT binding polypeptides are related rather than identical to the GAT V_H gene. Therefore to estimate the effect of nucleotide sequence divergence on the intensity of the hybridization signal, several V_H gene probes were hybridized to 2 ng of sucrose gradient-purified heavy chain RNA from the myeloma S107. For a messenger RNA present at 50 copies per cell, 2 ng is the amount of a 2 kb messenger sequence that would be present in 10 μg of total poly(A) RNA. All experimental conditions were identical to those described for the analysis of GAT V_H expression by T cells. Hybridization of C_κ, C_μ, and MOPC 21 V_H probes to varying amounts of F9-238.9 RNA provided a standard from which the effect on signal could be visually estimated (Figs. 6A and 6B). Hybridization of the S107 RNA to the VH11 probe, which shares 90% sequence identity, was reflected in three to fivefold loss of hybridization signal when compared to the completely identical S107 probe (Figs. 6C and 6D).

Furthermore, the VH14A probe which shared only 75% identity with the S107 RNA gave no detectable signal (Fig. 6E). Therefore, if the T cell hybridomas contained a moderately abundant sequence, less than 75-80% identical to the GAT V_H probe, this transcript would not have been detected.

Discussion

We wished to determine whether T and B cell receptors which bind the same antigen and share idiotypic determinants are encoded by similar V_H gene segments. The murine immune response to GAT is an ideal system in which to address this question. Therefore, we obtained and characterized a cDNA clone (pGAT50) containing the entire V_H coding sequence from F9-238.9, a GAT-specific B-cell hybridoma. The cDNA clone has been sequenced and is identical to the mRNA predicted from the 33 NH_2-terminal amino acid residues determined for the GAT-binding antibodies (22). The B cell response to GAT is relatively homogeneous and pGAT50 certainly should hybridize to mRNA encoding any of the five sequenced heavy chains present in antibodies which bind GAT. Therefore, if B and T cells responding to the same antigen use the V_H gene repertoire in a similar way, we would expect the GAT V_H probe to hybridize to transcripts from the GAT-specific T-cell clones.

Using pGAT50 as a probe, no hybridizing RNA species were detected on Northern blots of 12 different T cell RNAs. The GAT-specific T lymphocytes were of three different functional classes, T_S1, T_S2, and T_H. To ensure that the cells tested had maintained antigen-specific function, all lines were assayed for GAT-specific reactivity after expansion in culture and prior to RNA purification. Furthermore, positive control hybridizations with an H-2 class I probe argue that the RNA was intact and capable of hybridizing. Furthermore, although the GAT heavy chain transcript is only moderately abundant (Fig. 1), the GAT V_H sequence was detected in as little as 50 ng poly(A)-RNA from the GAT B hybridoma. Since 10 μg of each T-

hybridoma poly(A)-RNA was tested, we conclude that there must be at least 200 times (10 μg/50 ng) less GAT V_H-encoding RNA in the T hybridoma than in the B cell hybridoma. We estimate the detection limit of this analysis to be approximately 1.5 copies of GAT V_H sequence per T hybridoma.[2] A similar calculation leads to an estimated detection limit of 5-10 copies per cell for the two T cell lines tested. With the sensitivity of the Northern blots, we easily could have identified transcripts as abundant as the mRNA encoding μ heavy chain in B lymphomas, approximately 100 copies per cell (56). Furthermore, most mRNAs in the low abundance class are present in mammalian cells at 10-20 copies per cell (57), so we could have detected even a transcript in this class. Nevertheless, the T cells analyzed do not contain detectable levels of a messenger RNA similar to GAT V_H. This result is consistent with a report that a T_S clone which secretes a T_SF that binds phosphorylcholine and shares the predominant idiotype (TEPC15) of PC-binding antibodies, fails to transcribe or rearrange the TEPC15 V_H gene segment (58). Therefore, we conclude that shared idiotypic determinants need not imply transcription of highly similar V_H genes.

There are two limitations to the analysis we have presented. First, although unlikely, the homologous RNA could be present, with an average abundance of less than 1.5 molecules per cell. For example, despite the fact that antigen-specific function was assayed, it is possible that only 10% of the T lymphocytes were synthesizing antigen-receptor mRNA at the time of harvest. We consider this improbable, since function was tested directly after the cells were expanded in culture and 12 different cloned T cells were analyzed. However, if for example, the half-life of the T_SF protein were sufficiently long, mRNA need not have been continually present. Second, the hybridization signal we obtained is sensitive to the amount of similarity between the probe and the RNA sequence tested. Thus, if a GAT-specific T cell transcribed a V_H gene segment less than 80% similar to pGAT50,

this T-cell transcript probably would not have been detected. However, in the accompanying manuscript (49), we report the screening of T cell cDNA libraries directly with probes capable of hybridizing to virtually any known V_H sequence. These experiments demonstrate that T cells probably do not transcribe any gene segments from the B cell V_H repertoire.

Since there is no evidence for transcription of similar V_H gene segments by T and B cells responding to the same antigen, how does one explain the widespread occurrence of shared idiotypic determinants on these cells? First, it should be noted that serological cross-reactivity between molecules does not imply that they are highly similar in primary structure. In fact, there are numerous cases in which either complex antisera or monoclonal reagents detected cross reactions between molecules lacking extensive sequence similarity (59-62). For example, a monoclonal antibody raised against the Thy-1 antigen cross reacts with the S107 light chain variable region (60). Also, antibodies raised against insulin react with both insulin and with antibodies against the insulin receptor (61).

Generally there are two ways to consider the expression of idiotypic determinants by T lymphocytes. The cross reactions observed may be biologically irrelevant. In some cases, it is possible that T and B cell antigen receptors reported to share idiotypic determinants actually express no common serological specificity. For example, if the immunogen used to raise the anti-idiotypic reagent contained contaminating T cells or T cell-derived antigen-binding material, the resulting sera might detect different determinants on both B and T cells. In other cases, the detection of idiotype expression by T lymphocytes depended upon the use of complex antisera containing multiple serological specificities. In such complex sera, a minor subset of antibodies might recognize a determinant on both B and T cell receptors, even though these molecules share little or no structural similarity. Since the assay for T-cell idiotype is often the ability to disrupt T cell activity in vitro, perturbations

in T cell function due to a minor subset of antibodies would not be easily distinguishable from inhibition by the major components of the anti-idiotypic sera. No doubt other explanations are possible and no single rationale can account for every report of idiotype expression by T cells. However, the sharing of idiotypic determinants by B and T cells may be instead a biologically relevant phenomenon, reflecting a network or some other selection mechanism which acts similarly on both B and T cell antigen receptors. We favor this explanation for several reasons. First it has been demonstrated in several instances that monoclonal anti-idiotypic reagents can react with T lymphocytes, so in these cases, the reactivity cannot be due to minor components in a complex sera. These data formally demonstrate that T and B lymphocytes can share a single serologic determinant. Second, the presence of idiotypic determinants on T lymphocytes is generally correlated with antigen specificity. GAT-specific T cells express the CGAT and not the T15 idiotype, while some PC-specific T cells express T15. Third, in a number of cases, the expression of idiotype by T lymphocytes is linked to the genes encoding immunoglobulin heavy chain allotypes.

Although it is not certain how the presumptive selection mechanism operates, it is clear that such selective pressures do exist at least for the B cell compartment. For example, mice responding to phosphorylcholine primarily synthesize antibodies of the TEPC15 idiotype. However, if suppressed for TEPC15 production, the mice nevertheless can generate an idiotype-negative antibody response (63). Therefore, for B lymphocytes, the heterogeneity of receptors expressed in response to some antigens reflects both the diversity of the receptor repertoire and the selective forces which operate on it. Since T cells involved in many immune responses also express idiotypic determinants, it is likely that many anti-idiotypic sera recognize idiotopes involved in the selection or regulation of receptors. Finally, since T cell idiotype has been linked to the C_H locus, it has been concluded that the T cell receptor for antigen also is

encoded on chromosome 12. Alternatively, if the regulatory mechanism were dominated by B cell antigen receptors, the genes encoding the T cell antigen receptor would appear to map to chromosome 12 even though they are actually located elsewhere.

We conclude that T cells and B cells which recognize the same antigen and share idiotypic determinants, need not transcribe similar V_H gene segments. Understanding the structural and genetic bases for idiotype expression by T cells awaits a thorough characterization of the antigen-binding molecules themselves and the genes which encode them.

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Footnotes

[1]Abbreviations used in this paper: AMV, avian myeloblastosis virus; C, constant region; CGAT, predominant idiotype in murine response to GAT; D, diversity gene segment; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; dT, deoxythymidine monophosphate; GA, synthetic polymer of glutamic acid^{50}:alanine^{50}; GAT, glutamic acid^{60}:alanine^{30}:tyrosine^{10}; GT, glutamic acid^{50}:tyrosine^{50}; H, immunoglobulin heavy chain; HAT, medium containing hypoxanthine, aminopterin and thymidine; Ig, immunoglobulin; IL-2, interleukin 2 or T cell growth factor; J, joining gene segment; κ, kappa light chain; Kb,kilobase (1000 nucleotides); PC, phosphorylcholine; T_H, helper T cell; T_S, suppressor T cell; T_SF, T cell suppressor factor; UT, untranslated; V, variable region.

[2]By comparing the signal obtained with J_H, MOPC21 V_H, GAT50 V_H, and C_μ probes hybridized to F9-238.9 RNA and purified myeloma RNAs, we estimate that approximately 0.2% of the F9-238.9 poly(A)-RNA encodes the GAT Ig heavy chain (Kraig and Kronenberg, unpublished observation). The 5' untranslated (5' UT) region plus V_H comprise 21% = (500/2400) of the mass of the entire μ mRNA, therefore, the V_H plus 5' UT should be present at 0.04% of the mass of the F9-238.9 poly(A)-RNA. Since a sequence this abundant could be detected in 50 ng of B cell RNA (Fig. 4, lane 3) and since 10 μg of each T hybridoma poly(A)-RNA was tested, we could have detected an homologous V_H sequence present in T cells at 0.00021% of the mass of the poly(A)-RNA. Assuming 0.2 pg poly(A)-RNA per T cell (49), we could have detected as little as 4×10^{-7} pg V_H/cell. One molecule of 5' UT-V_H weighs 2.76×10^{-7} pg = (500 nucleotides) (330 daltons/nucleotide) (1.67×10^{-12} pg/dalton). Therefore the calculated detection limit is 1.5 molecules/cell = (4×10^{-7} pg detectable/cell)/(2.76×10^{-7} pg/5' UT-V_H). This number is a best estimate, but there is a degree of uncertainty in determining both the percentage of the F9-238.9 poly(A)-RNA which encodes the GAT immunoglobulin heavy chain and the amount of poly(A)-RNA per cell in the T cells.

Footnote 2 (continued)

Since less RNA from the T_H cell lines was tested, the detection limit is two to threefold higher than the detection limit for the T cell hybridomas.

FIG. 1. Hybridization of a probe containing the four germline J_H gene segments to a Northern blot of poly(A)-RNA from the F9-238.9 GAT B-cell hybridoma. The migration distances of the RNA molecular weight standards and their sizes in kilobases are indicated. The lower band contains MOPC21-γ RNA while the upper band contains GAT V_H-μ. For an additional comparison, see Figs. 6A and 6B.


5
2.9
2
1.54

FIG. 2. Ologonucleotide probes. The GAT V_H amino acid sequence, the predicted mRNA sequences and the pentadecamers synthesized as probes complementary to the mRNA are shown.

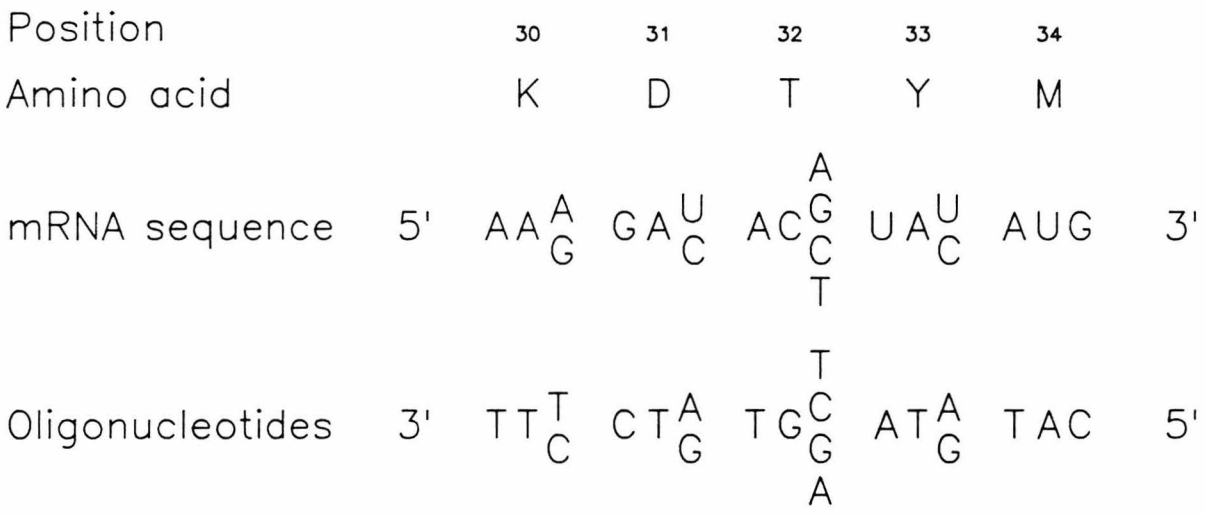

Position
30
31
32
33
34
Amino acid
K
D
T
Y
M
mRNA sequence
5'
AA A/G
GA U/C
AC A/G/C/T
UA U/C
AUG
3'
Oligonucleotides
3'
TT T/C
CT A/G
TG T/C/G/A
AT A/G
TAC
5'

Figure 2

FIG. 3. Characterization of pGAT50. The pGAT50 cDNA clone is shown with the recognition sites for the restriction endonucleases, Pst I (), PvuII (), and StuI (), indicated. The specified restriction fragments from pGAT50 and pGAT40 (*), a second cDNA clone containing only part of the GAT V_H sequence, were subcloned into M13mp8, a bacteriophage vector, and sequenced by the dideoxy method (42) in the direction indicated by the arrows. The sequence of pGAT50 was confirmed and extended by the Maxam-Gilbert method (43) from the StuI site, as shown.

The resulting DNA sequence of pGAT50 and its translation into protein sequence are given. The position of the pentadecamer used as a hybridization probe is underscored. Vertical lines delineate the boundaries of 5' UT, L, V_H, D, and J_H.

```
  1  TTCTGGTAGTTCCANGTGAAGGAAATGGTGCTGGGCAGGAPGTCCCGGGCCAPGCAGCCC   60

-19                                                  M  K  C  S    -16
 61  ATGAGCCCTATCTTCTCTACAGACACTGAATCTCAAGGTCCTTACAATGAAATGCAGC    118

-15   W  V  I  F  F  L  M  A  V  V  T  G  V  N  S | E  V  Q  L  Q     5
119  TGGGTTATCTTCTTCCTGATGGCAGTGGTTACAGGGGTCAATTCAGAGGTTCAGCTGCAG  178

  6   Q  S  G  A  E  L  V  K  P  G  A  S  V  K  L  S  C  T  A  S    25
179  CAGTCTGGGGCAGAGCTTGTGAAGCCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTCT  238

 26   G  F  N  I  K  D  T  Y  M  H  W  V  K  Q  R  P  E  Q  G  L    45
239  GGCTTCAACATTAAAGACACCTATATGCACTGGGTGAAGCAGAGGCCTGAACAGGGCCTG  298

 46   E  W  I  G  R  I  D  P  A  N  G  N  T  K  Y  D  P  K  F  Q    65
299  GAGTGGATTGGAAGGATTGATCCTGCGAATGGTAATACTAAATATGACCCGAAGTTCCAG  358

 66   G  K  A  T  I  T  A  D  T  S  S  N  T  A  Y  L  Q  L  S  S    85
359  GGCAAGGCCACTATAACAGCAGACACATCCTCCAACACAGCCTACCTGCAGCTCAGCAGC  418

 86   L  T  S  E  D  T  A  V  Y  Y  C  A  S | Y  Y  G  S  S  Y | Y   105
419  CTGACATCTGAGGACACTGCCGTCTATTATTGTGCTAGTTACTACGGTAGTAGCTACTAC  478

106   Y  W  G  Q  G  T  T  L  T  V  S  S | E  S  Q  S  F  P  N  V   125
479  TACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGAGAGTCAGTCCTTCCCAAATGTC  538

126   F  P  L  V  S  C  E  S  P  L  S  D  K  N  L  V               140
539  TTCCCCCTCGTCTCCTGCGAGAGCCCCCTGTCTGATAAGAATCTGGT                585
```

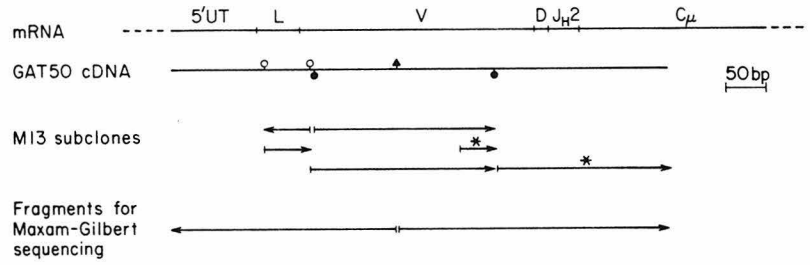


Figure 3

FIG. 4. Hybridization of Northern blot containing RNA from GAT-specific T and B cells with the pGAT50 probe. Varying amounts of poly(A)-RNA from F9-238.9, the GAT-B hybridoma, ten μg of poly(A)-T cell RNA from the T-cell hybridomas were loaded as follows: lane 4, 301D4A5; lane 5, 342B1.11; lane 6, 365C6.4; lane 7, 258C4.4; lane 8, 367A5.2; lane 9, 368B1.5; lane 10, BW5147; lane 11, 145F3511; lane 12, 301A2.3; lane 13, 469B5.5; lane 14, 372B3.5; and lane 15, 372D6.5. The positions, i.e., 301A2.3, and sizes (in kilobases) of the rRNA markers are indicated.

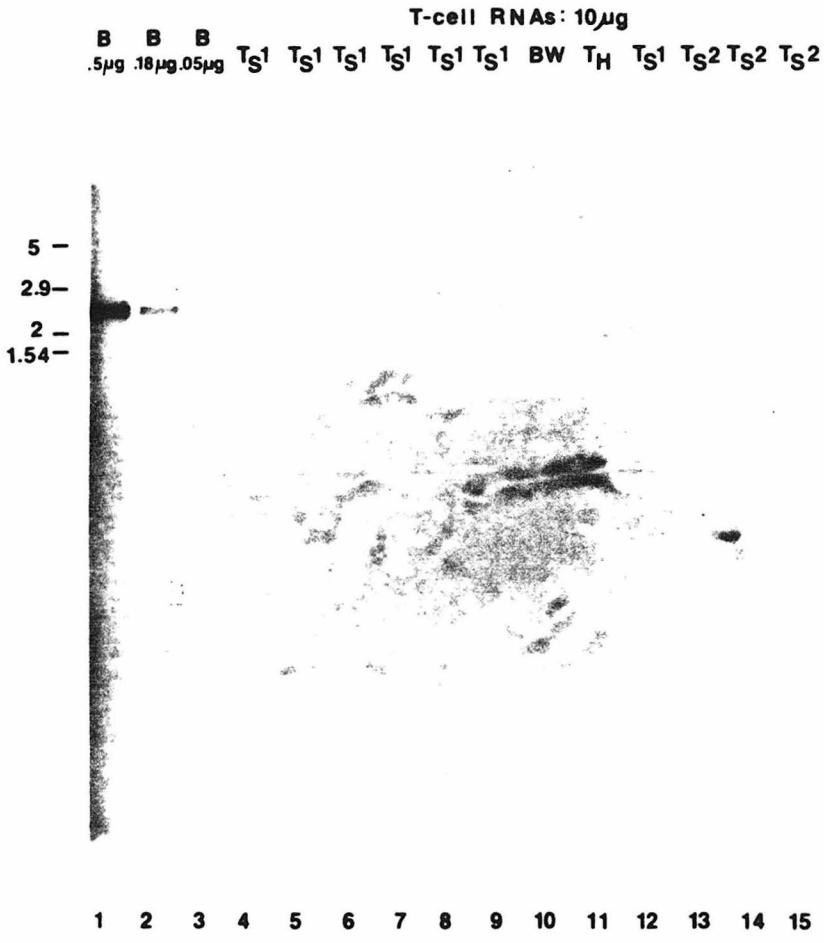

T-cell RNAs: 10µg
B .5µg
B .18µg
B .05µg
T_S^1
T_S^1
T_S^1
T_S^1
T_S^1
T_S^1
BW
T_H
T_S^1
T_S^2
T_S^2
T_S^2
5
2.9
2
1.54
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIG. 5. Hybridization of Northern blot of T cell RNAs with H-2 class I probe. The blot used in Fig. 4 was rehybridized with pH-II2A, a murine class I probe. Residual hybridization of pGAT50 to the B cell RNA is observed. The positions and sizes (in kilobases) of the rRNA markers are indicated.

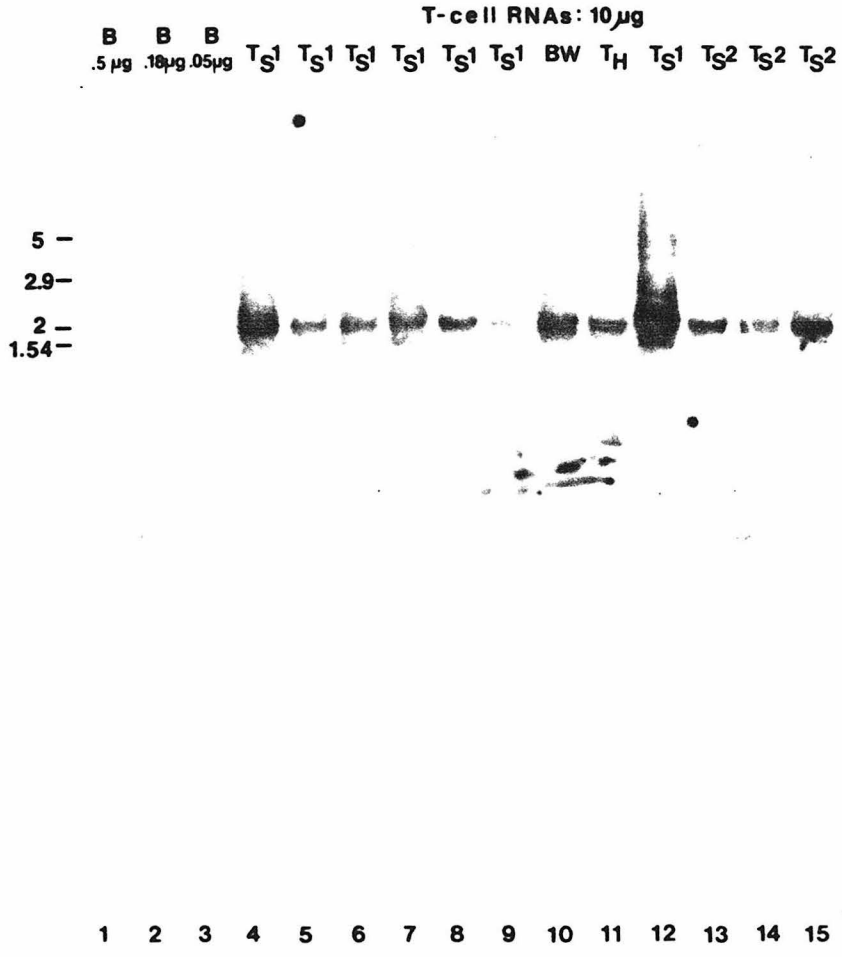
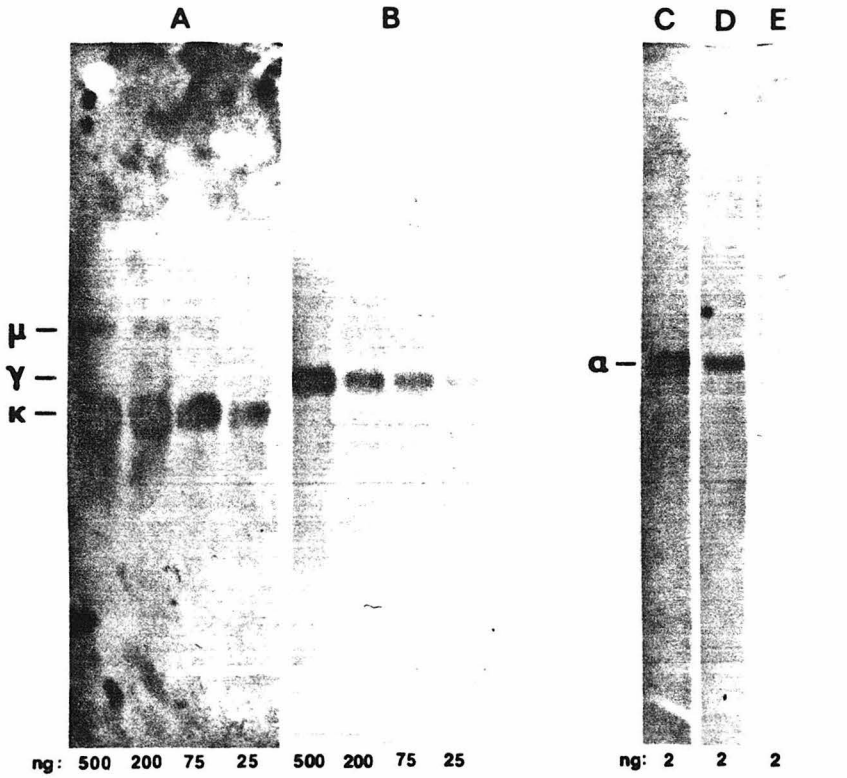

T-cell RNAs: 10μg
B .5 μg
B .18μg
B .05μg
TS1
TS1
TS1
TS1
TS1
TS1
BW
TH
TS1
TS2
TS2
TS2
5
2.9
2
1.54
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIG. 6. Effect of nucleotide sequence divergence upon hybridization signal. A. Hybridization of a C_{μ} and C_{κ} probe to various amounts of F9-238.9 RNA. B. Hybridization of the MOPC21 V_H cDNA to various amounts of F9-238.9 RNA. C. S107 RNA hybridized with the S107 V_H probe. D. S107 RNA hybridized with the VH11 probe (90% similar). E. S107 RNA hybridized with the VH14A probe (75% similar).



I. F9-238.9 RNA

II. S107 H-chain RNA

TABLE I

Characteristics of GAT-T Cell Clones

T cell clones	Strain of normal T-cell parent	Fusion partner	Immunogen	T cell functional class	Type of factor	CGAT Idiotype	Antigen specificity		
							GAT	GA	GT
258C4.4	DBA/1	BW5147	GAT	suppressor	T_SF_1	+	+	+	-
342B1.11	B10.S	BW5147	GAT	suppressor	T_SF_1	+	+	-	+
365C6.4	B10.S	BW5147	GAT	suppressor	T_SF_1		+		
367A5.2	CBA	BW5147	GT	suppressor	T_SF_1				+
368B1.5	BALB/c	BW5147	GT	suppressor	T_SF_1	+	+	-	+
Fc301D4.5	B10.M	BW5147	GT	suppressor	T_SF_1				+
Fc301A2.3	B10.M	BW5147	GT	suppressor	T_SF_1				+
469B5.5	B10.A	BW5147	GT-T_SF+GT	suppressor	T_SF_2	+	+	-	+
372B3.5	B10	BW5147	GAT-MØ[a]	suppressor	T_SF_1	+	+	-	+
372D6.5	B10	BW5147	GAT-MØ[a]	suppressor	T_SF_2	+	+	-	+
145F3511	B10.A	BW5147	GAT	helper[b]			+		
BB02'	C57BL/10	none	GAT	helper			+		
BD01'	(B10xB10.D2)F_1	none	GAT	helper			+		

[a]Treated as neonates with GAT coupled to macrophages.

[b]Cells synthesize IL-2 in response to antigen and I region syngeneic antigen presenting cells.

Chapter 5

THREE T CELL HYBRIDS DO NOT EXPRESS DETECTABLE HEAVY CHAIN VARIABLE GENE TRANSCRIPTS

This chapter has been accepted for publication in the Journal of Experimental Medicine. Figures 6 and 7 have been published previously in Isolation, Characterization and Utililzation of T Lymphocyte Clones, C. Garrison Fathman and Frank Fitch, editors. Academic Press, New York. p. 467 and are reprinted here with permission of the publisher.

THREE T-CELL HYBRIDOMAS DO NOT CONTAIN DETECTABLE HEAVY CHAIN VARIABLE GENE TRANSCRIPTS*

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Running Head: T-Cell V Genes

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Footnotes

[1] Abbreviations used in this paper: cDNA, DNA complementary to mRNA; D, diversity gene segment; GAT, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GT, L-glutamic acid^{50}-L-tyrosine^{50}; HGG, human gamma globulin; J_H, heavy chain joining gene segment; kb, kilobase; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; NP, 4-hydroxy-3-nitrophenyl acetyl; PC, phosphorylcholine; V_H, heavy chain variable; V_κ, kappa light chain variable; V_λ, lambda light chain variable.

[2] A number of measurements have indicated that most mammalian cells, including lymphocytes, contain about 10^4 different sequences or species of mRNA of average length 2×10^3 nucleotides (48-51). Thus, about 2×10^7 base pairs ($= 10^4 \times 2 \times 10^3$) of genomic DNA are transcribed into mRNA. The random chance of any one of the four nucleotides occurring at a particular place in a DNA sequence is 1/4; therefore, if we ignore the effects of base composition and nearest neighbors, the probability that an 11-nucleotide sequence will occur is $(1/4)^{11} = 2.5 \times 10^{-7}$. Multiplying the probability of occurrence for the undecamer by the number of nucleotides in the mRNA gives the number of different mRNA species expected to be perfectly complementary to the oligonucleotide, $(2 \times 10^7)(2.5 \times 10^{-7}) = 5$.

There is considerable controversy as to whether or not the genes encoding the T-cell antigen receptor are homologous to immunoglobulin gene segments. Results from a number of different experiments have been interpreted as evidence that T lymphocytes utilize heavy chain variable (V_H[1]) regions to bind specifically to antigen. For example, some anti-idiotypic and anti-V_H framework sera interfere with T-cell function and/or bind to antigen-specific factors secreted by T cells (1-3). In several cases, the gene encoding the cross-reactive determinant expressed by the T cells is linked to the immunoglobulin heavy chain gene cluster (4-9). Also, the genes encoding a series of T-cell alloantigens have been mapped to chromosome 12, between the C_H gene locus, Igh-1, and the prealbumin gene (9, 10). Recently, these alloantigens have been detected on antigen-binding factors secreted by T cells (11-13). It has been proposed that the antigenic determinants encoded by C_H-linked genes are T-cell isotypes which may be expressed in conjunction with V_H gene segments (10, 14). Furthermore, some T lymphocytes contain rearranged J_H gene segments or a C_μ transcript (15-23). This may indicate that the mechanisms controlling V_H-D-J_H joining and immunoglobulin transcription also operate upon homologous sequences in the synthesis of T-cell antigen-binding receptors.

Experiments which report the expression of V_H serologic determinants by T lymphocytes have provided the most extensive and convincing data in support of V_H gene transcription by T cells. However, the serologic data are indirect, and there are three possible ways to interpret them. First, T and B cells responding to the same antigen may express highly similar or identical V_H gene segments. It should be noted that in several experimental systems the receptor synthesized by T cells responding to an antigen does not share all the serologic determinants present on the immunoglobulin synthesized by B cells responding to the same antigen (1, 24). In addition, we and others have demonstrated that idiotype-positive T and B lymphocytes which respond to the same antigen do not transcribe highly similar V_H gene

segments (25, 26). Second, it is possible that T cells use the repertoire of V_H genes differently than B cells do. This might occur because T lymphocytes do not express light chain genes (3, 4) or because T cells recognize antigen in the context of syngeneic MHC gene products. If this were true, then T cells responding to an antigen may transcribe V_H gene segments which have limited structural similarity to those transcribed in B cells responding to the same antigen, although these different V_H gene products could share some idiotopes. Finally, it is possible that the V_H cross-reactive determinants present on T cells and T-cell factors are not the products of V_H genes.

In this paper, we report our attempts to determine whether any V_H gene segments are expressed in T lymphocytes. cDNA libraries were constructed from a suppressor T-cell hybridoma specific for the synthetic polypeptide GAT, and from two helper T-cell hybridomas, one specific for HGG and the second responding to KLH. The cDNA libraries were hybridized with two sets of probes; each set capable of detecting a wide range of V_H gene segments. In constructing the probes, no assumptions were made concerning the degree of homology between the B-cell heavy chain variable regions binding GAT, HGG, or KLH and V_H gene transcripts which might be present in the T-cell hybridomas. One set of probes was a synthetic oligonucleotide complementary to a conserved sequence found at the 3' end of many mouse V_H gene segments and a single-stranded cDNA synthesized primarily from the heavy chain variable genes present in spleen RNA. The second set of probes was two cloned V_H gene segments, one from the V_HII gene subgroup and one from the V_HIII gene subgroup. The cDNA libraries were sufficiently large so that the chance of detecting a sequence found in the nonabundant messenger RNA class (10-20 copies per cell) was excellent. Since no V_H-containing cDNA colonies were found, we conclude that V_H gene segments are not likely to encode the T-cell antigen-binding receptor.

Methods

RNA Preparation. T-cell hybridomas were grown in liquid culture and harvested. The cell pellets were lysed in guanidinium thiocyanate, and the RNA was prepared by centrifugation through a cushion of cesium chloride (27). The percent yield and the amount of RNA per cell were estimated using a recovery marker as previously described (20). RNA was similarly prepared from spleens of 6-month-old BALB/c Cum mice. Poly(A)$^+$ RNA was purified by two cycles of oligo(dT)-cellulose chromatography (28).

cDNA Synthesis. Double-stranded cDNA was synthesized as described (29, 30). First strand synthesis was initiated by random priming using sheared calf thymus DNA (31). The double-stranded cDNA was fractionated by gel filtration and the material ranging in size from 400 to 1500 base pairs was pooled. The average length of the cDNA was approximately 800 base pairs. The cDNA was cloned into the Pst I site of the tetracycline-resistant plasmid pBR322 by annealing dC-tailed cDNA to dG-tailed vector (32). Bacterial strain MC1061 (33) was transformed with cDNA and the transformants were selected with tetracycline (34). We obtained approximately 10^6 colonies per μg of cDNA. Transformation with vector alone (dG-tailed pBR322) yielded a 2% background.

Synthetic Oligonucleotides. Two undecamers were synthesized separately by Dr. S. Horvath (California Institute of Technology) by the phosphite coupling method (35, 36) (Fig. 1). The sequences were verified by the method of Maxam and Gilbert (37). The oligonucleotide probes were labeled with 5'-[γ-^{32}P]dATP to a specific activity of greater than 2 μCi/pmol using T4 polynucleotide kinase (38). Filters were prehybridized and hybridized with the radioactive oligonucleotides at 4°C as previously described (25). The filters were washed with several changes of 5X SET (0.75 M NaCl/0.15 M Tris pH 8.0/5 mM EDTA) with 0.1% sodium pyrophosphate between 12 and 20°C and were then exposed to film.

Specifically-Primed cDNA Made from Spleen RNA. The synthesis of the J_H-primed spleen cDNA probe is illustrated in Fig. 2. The primer was prepared by purifying a 2 kb Hpa II fragment containing the four J_H coding sequences from a subclone derived from the bacteriophage lambda clone ChSpμ27 which contains germline BALB/c DNA. This 2-kb fragment was then digested with the restriction enzymes Dde I, Hae III, Pst I, and Rsa I. This results in a number of restriction fragments, including four which contain part of the J_H coding sequences (21) (Fig. 3). The restriction fragments were denatured by boiling and annealed to poly(A)$^+$ RNA from spleen. cDNA synthesis primed with the annealed J_H fragments was carried out as described (29). The concentration of α-^{32}P-labeled and unlabeled deoxynucleotide triphosphates was adjusted so that the synthesized material had a specific activity of 2-3 x 10^8 cpm/μg. RNA in the reaction was hydrolyzed with alkali and the single-stranded cDNA was separated from unincorporated nucleotides by gel filtration. The yield of cDNA was about 0.5% the mass of spleen RNA in the reaction; a fourfold stimulation over a reaction with no added primer. Filters were prehybridized at 50°C and hybridized at the same temperature with 5 ng/ml of J_H-primed cDNA for 48 h. Conditions were otherwise as previously described (25). Filters were washed at 50°C in several changes of 5X SET/0.1% sodium pyrophosphate/0.1% SDS before exposure to film.

V Region Probes. The plasmid p107V1 contains the entire gene segment coding for the heavy chain variable region expressed in the S107 myeloma (39). The V_H gene can be separated from the pBR322 vector DNA by digestion with Pst I. The plasmid pVH_3 obtained by the laboratory of Dr. Sam Strober, was provided by Dr. Michael McGrath, Stanford University. This plasmid has a 1 kb Bam HI fragment which contains the heavy chain variable gene expressed by the BCL1 lymphoma (40). The 1 kb Bam HI fragment of pVH_3 and the 445 base pair Pst I fragment of p107V1 were nick translated (41) to a specific activity of 1-8 x 10^8 cpm/μg. Filters hybridized

with these probes were handled as described for the J_H-primed cDNA except the probe was present at a concentration of 0.2-1.0 ng/ml.

Colony Hybridization. Nitrocellulose filters (HATF 13750, Millipore, Bedford, MA) were replica plated and prepared for in situ hybridization as described (42). Duplicate filters were annealed with each probe. For each screening, a positive control filter with colonies containing a heavy chain variable gene segment (MOPC21) was hybridized in parallel (Fig. 4).

Southern Blots. Plasmid DNA was prepared from clones isolated from the cDNA libraries. This DNA was digested with various restriction endonucleases, separated by molecular weights in 1% (w/v) agarose gels and transferred to nitrocellulose (43). The filters were then hybridized with the synthetic oligonucleotides or the J_H-primed spleen cDNA as described above.

DNA Sequencing. Restriction fragments were labeled at the 5' end with ^{32}P-γ-dATP using polynucleotide kinase (37) or labeled at the 3' end with ^{32}P-α-cordycepin-5'-triphosphate using terminal deoxynucleotidyl transferase (44). The labeled fragments were cut internally with a second enzyme and those isolated fragments were sequenced according to the method of Maxam and Gilbert (37).

Results

cDNA libraries were constructed from three different T-cell hybridomas. Some features of the three hybrid cell lines are summarized in Table I. These cells were chosen for several reasons. First, they have retained function which is antigen specific, and in two cases MHC restricted. Second, they grow continuously in the absence of irradiated feeder spleen cells which, if present, could contribute contaminating immunoglobulin sequences to the T-cell RNA preparations (21). Third, the hybridomas are specific for structurally unrelated antigens and are therefore likely to employ rather different antigen-binding receptors. If T lymphocytes do

transcribe V_H gene segments, this should increase the probability that the probes will have sufficient homology to hybridize with a V_H gene transcript from at least one of the lines. Finally, two hybridomas help B cells secrete antibody and the third secretes a specific suppressor factor. Since most reports of idiotype expression by T lymphocytes involve the helper or suppressor functional subclasses, these types of cells may be best suited for the detection of V_H transcripts (2, 3, 7). To detect virtually any V_H gene transcript present in these cells, we employed two experimental strategies for screening the cDNA libraries.

Hybridization with Synthetic Oligonucleotides and J_H-Primed cDNA. In the first attempt, two types of probes, synthetic oligonucleotides and a J_H-primed cDNA, were used to screen the libraries. Each probe will hybridize to a variety of V_H gene segments, as well as to some sequences that do not contain V_H genes. The frequency of such non-V_H hybridizing sequences is low, so that a clone hybridizing with both types of probes probably contains a V_H gene segment.

The sequences of the eleven-base synthetic oligonucleotides are 5' GCA CAG TAA/G TA 3' (Fig. 1). These probes are complementary to a highly conserved sequence found at the 3' end (amino acids 95-98) of mouse heavy chain variable region gene segments. A sequence perfectly complementary to either oligonucleotide is found in 50% (17/34) of the murine V_H genes for which DNA sequence data are available. The degree of homology of the cloned murine V_H gene segments to the oligonucleotides is listed in Table II. Furthermore, in those cases for which no DNA sequence but amino acid sequence data are available, 78% (18/23) of the mouse immunoglobulin variable regions have tyr-tyr-cys-ala at positions 95-98, and therefore may share complete homology to one of the probes (45, 46). However, the oligonucleotide is not long enough to identify unambiguously a V_H gene segment. Given the number of nucleotides of genomic DNA transcribed into RNA, and the random chance of occurrence of an 11-nucleotide sequence, we calculate that any

mouse cell, whether it synthesizes immunoglobulin or not, should contain about five species of messenger RNA which will hybridize with each oligonucleotide.[2]

The second type of probe was a cDNA synthesized from spleen RNA (Fig. 2). The spleen contains a relatively high proportion of B cells which should express many different immunoglobulin heavy chains. Therefore, it was possible to use J_H DNA as a primer to stimulate the synthesis of a radioactive single-stranded cDNA complementary to many V_H genes. Using either Southern blots or hybridization to cloned DNA spotted onto nitrocellulose filters, the specifically primed cDNA hybridized to several V_H gene segments tested. These include T15, MOPC21, V_HB2, and V14A (Fig. 5). Since two of the hybridizing gene segments, T15 and V_HB2, share less than 60% homology (Table II), this probe should hybridize to a large number of different V_H sequences. In addition, the specifically primed cDNA does hybridize to a few cloned DNA segments which do not contain V_H genes (M. Kronenberg, unpublished observations). There are two explanations for hybridization to sequences lacking V_H genes: 1) There is a significant amount of cDNA synthesis in the absence of added primer (see Methods) which should not be enriched for immunoglobulin sequences. 2) The primer DNA is a mixture of restriction fragments from both the J_H gene segments and the intervening and nearby flanking sequences. Some of the fragments from the noncoding DNA may prime cDNA synthesis from nonimmunoglobulin sequences in spleen RNA. It is possible that some of the nonimmunoglobulin sequences which hybridize with the probe are repeated DNA sequences which are transcribed abundantly in spleen cells. We have not, however, characterized these hybridizing sequences.

To demonstrate that these probes can detect a V_H sequence we screened a cDNA library made with RNA extracted from a B-cell hybridoma (25). A colony which hybridized with both the oligonucleotide and the J_H-primed cDNA (21) was characterized further. The nucleotide sequence of this cDNA clone (Fig. 6) indicates

that it encodes the MOPC21 heavy chain variable region synthesized by the P3-X63-Ag8 myeloma parent cell. The clone includes almost the entire V_H gene segment beginning at the codon for amino acid 2 as well as the entire D and J_H4 gene segments.

Having determined that these probes were able to detect B-cell V_H gene segments, we screened the three T-cell cDNA libraries with the synthetic undecamers. The filters were hybridized and washed under conditions such that 11/11 homology was required to give a positive signal. Fifty-four positive colonies were found (Table III). A single filter containing three hybridizing colonies is shown (Fig. 7). The frequency of positives was low, indicating that these cells do not contain abundant RNA molecules with sequences complementary to the undecamers. Northern blots hybridized with the synthetic oligonucleotide gave a similar result (M. Kronenberg, unpublished observations). In fourteen cases, the colony which hybridized with the oligonucleotide was isolated and the plasmid DNA prepared from the bacterial clone. Southern blots of this plasmid DNA also hybridized with the oligonucleotide, thereby confirming the colony hybridization results (Fig. 8 and unpublished observations).

Following hybridization with the oligonucleotides, the three cDNA libraries were screened with the J_H-primed cDNA. To test for colonies homologous to both probes, all filters which contained a colony that annealed with the oligonucleotide were hybridized with the cDNA probe. The frequency of positive colonies was fifteen- to fortyfold higher with the J_H-primed spleen cDNA than was obtained with the synthetic probe (Table III). However, none of the colonies which hybridized with the spleen cDNA also hybridized with the oligonucleotide (Fig. 7). Plasmid DNA isolated from fourteen colonies which hybridized with the undecamer was also tested with this probe. None of the isolated plasmid DNA hybridized with the J_H-primed cDNA (Fig. 8).

Hybridization with Cloned V_H DNA Sequences. Using cloned V_H DNA probes and hybridization conditions of decreased stringency, it is possible to detect cDNA colonies containing V_H genes which are only 55-60% homologous to the probe (S. Crews, unpublished observations, Fig. 4). We therefore screened the three T-cell cDNA libraries with V_H probes from the S107 (V_H subgroup III) and BCL1 (V_H subgroup II) tumors. These were chosen because the complete DNA sequences of these V_H gene segments are available and because fragments of the appropriate sizes are easily prepared without contaminating vector or much flanking DNA. However, there were no colonies in the three T-cell cDNA libraries which hybridized with either of these probes. A positive control filter with colonies containing the MOPC21 V_H gene segment hybridized with pVH_3, which contains the V_H gene expressed in BCL1 (Fig. 4). The MOPC21 and pVH_3 V_H gene segments share only 58% homology. Table II indicates that the V_H gene segments for which nucleotide sequences are available have greater than 56% homology to at least one of our probes. Therefore, it is likely that we could have detected all of these V_H genes.

Discussion

We have utilized several strategies to evaluate whether T cells express any V_H gene segments. To accomplish this, we had to construct DNA probes capable of detecting many different V_H genes. The Northern blot hybridization is the most direct method to test for a particular transcript present even at the level of a single copy per cell (20). For the detection of possible V_H transcripts in T cells, we decided instead to screen large cDNA libraries. There are two reasons for doing this. First, we have found that it is possible to detect V_H sequences less than 60% homologous to the probe in hybridizations to cDNA colonies (Fig. 1), while greater than 80% homology is required when hybridizing under conditions of moderate stringency to Northern blots (25). This difference may reflect a number of factors, including the

concentration of the filter-bound nucleic acid. In addition, we have achieved a greater hybridization signal to filter bound DNA as opposed to RNA, even when both nucleic acids contained identical sequences electrophoresed in parallel and hybridized to the same probe in the presence of 50% formamide (M. Kronenberg, unpublished observations). Second, the initial strategy to detect a V_H gene segment involved the use of two probes which could hybridize to a number of sequences, including those not containing V_H genes. Since there are multiple sequences present in the poly(A)$^+$ RNA which hybridize to these probes, we detect diffuse smears rather than discrete bands on Northern blots. By contrast, each cloned cDNA in the library is physically separate and could be analyzed individually for the possibly rare sequences which hybridize with both probes.

The cDNA libraries were screened with a synthetic oligonucleotide and a J_H-primed cDNA made from spleen RNA. This method has been characterized extensively by test hybridizations (21) (Figs. 4, 5) and by the cloning and sequencing of the MOPC21 V_H cDNA from a B-cell hybridoma (Fig. 6). Of the approximately 1.2 x 10^6 colonies from three T-cell cDNA libraries hybridized with the synthetic oligonucleotide, fifty-four clones were positive. Because of the relatively short length of the probe, we would have predicted, on statistical grounds, the existence of such colonies even in cDNA made from nonlymphoid RNA. None of these colonies also annealed with the specifically-primed spleen cDNA when tested in situ (54 cases) or after Southern blotting the purified plasmid DNA (14 cases). A relatively large number of colonies did, however, hybridize with the spleen cDNA probe. The identification of colonies which hybridized with either one of the two probes provided an internal control and indicates that there was no technical problem with the screening which would have prevented us from identifying clones of interest. Positive control hybridizations with V_H-containing colonies support this conclusion. Since no colonies hybridized with both probes, we conclude that V_H gene segments are absent

from these libraries. Hybridization with cloned V_H DNA gene segments under conditions whereby sequences less than 60% homologous to the probe could be detected constituted a second, independent test for the presence of V_H genes. By hybridizing the cDNA libraries with just two V_H sequences from different heavy chain subgroups, we should be able to detect all of the well-characterized V_H genes. No T-cell cDNA colonies hybridized with these probes, confirming the result obtained by the first method.

While B lymphocytes expressing cell-surface IgM contain 100-200 copies of C_μ RNA per cell (17, 47), a messenger RNA for the T-cell receptor may not be this prevalent. The bulk of the 5,000-15,000 sequences found in most eukaryotic cells, including lymphocytes, are in the low abundance class (10-20 copies/cell) (48-51). Our calculations indicate that we had a good chance of detecting V_H sequences transcribed at this level (Table IV). For example, if there is a V_H sequence homologous to the probes and present at 10 copies per cell, the probability of detection was 77% for the AODK 10.4 cDNA library, 88% for the AODH 7.1 cDNA library, and greater than 99% for the 395A4.4 library. The probabilities of detecting a sequence present at 15 or 20 copies per cell are higher. If each library construction and screening were an independent event, then the overall probability of not obtaining a V_H clone which is present at 10 copies per cell becomes extremely low (0.23 x 0.12 x 0.01 = 0.00028). This analysis cannot exclude the possibility of expression of V_H genes at one or even a few copies per cell. However, there is some indication that these hybrids synthesize a significant amount of receptor protein. The T-cell hybridoma AODH 7.1 binds antigen avidly in the presence of the proper antigen-presenting cells and almost all the cells in the culture retain this ability (J. Kappler and P. Marrack, unpublished observations). In addition, the 395A4.4 hybridoma constitutively synthesizes both an antigen-binding suppressor factor and an antigen-binding receptor. We therefore consider it unlikely that the receptor mRNA could be

present at an extremely low-copy number.

The calculated detection limit depends on a formula which assumes that the probability of detecting a cDNA is solely a function of the abundance of its RNA template in the population (52, 53). This will be true only if the synthesis and cloning of cDNAs uses all templates with equal efficiency. Although factors such as secondary structure of an RNA (54) may influence the ultimate cloning efficiency, there does not appear to be a selection against cloning of heavy chain variable region cDNA. In addition, since the bulk of the first strand of cDNA synthesis is shorter than the average 2-kb length of an mRNA, there is an intrinsic bias towards obtaining cDNA clones containing sequences close to the point of initiation of synthesis. By using a sheared calf thymus DNA primer to randomly initiate cDNA synthesis at many points along the RNA templates, as opposed to an oligo(dT) primer which will initiate synthesis at the 3' end, we eliminated any bias towards obtaining clones corresponding to only one end of the messenger RNA. Given these considerations, we feel justified in using the formula shown in Table IV. Finally, in order to calculate the probability of cloning a particular messenger RNA, we needed to estimate the amount of poly(A)$^+$ RNA per cell. Each of the hybrid cells contains between 5 and 10 pg of total RNA (Table IV). We assumed that 0.3 pg of this total is in the poly(A)$^+$ fraction, although our actual yield was substantially lower, between 0.05 and 0.15 pg per cell. Poly(A)$^+$ RNA selected by oligo(dT)-cellulose chromatography will contain a residual poly(A)$^-$ component which is mostly ribosomal RNA. Since this material may give rise to a proportionate fraction of the cDNA colonies in Table IV, we corrected N, the number of colonies screened, to account for the contaminating poly(A)$^-$ RNA.

The calculations are based upon reasonable estimates of the purity of the poly(A)$^+$ RNA and the amount present per cell. However, if we assume there is somewhat more than 0.3 pg of poly(A)$^+$ RNA per cell, or if the poly(A)$^+$ fraction of the RNA preparation is less than 75% of the total mass, the basic conclusion remains

valid. For example, if the oligo(dT)-passaged RNA were only 50% pure, the probability of detecting a homologous V_H sequence present at 10 copies per cell becomes 63% for the AODK 10.4 cDNA library, 75% for the AODH 7.1 library, and greater than 99% for the 395A4.4 library.

Although unlikely, it is possible that the three T lymphocytes might express a V_H gene which could not be detected by either of our two screening methods. The DNA sequence homology of mouse V_H gene segments to our probes is presented in Table II. The J_H-primed spleen cDNA hybridized with all of the five V_H gene segments tested (21). In addition, homology with the undecamers does not appear to be restricted to any particular type of heavy chain. Some murine V_H genes coding for proteins in subgroups I, II, and III, as well as three out of four human V_H gene segments (all subgroup III) have complete homology to these probes (55, 56). Even two mouse germline V_λ gene segments have 10/11 matching nucleotides (57, 58). However, the synthetic oligonucleotides will not hybridize with half of the known murine V_H gene segments. In principle, none of these genes would have been detected by our first method. However, all of the V_H gene segments listed in Table II are greater than 56% homologous to one of the two cloned V_H probes and probably could be detected by the second method.

Estimates of the ability of the probes to detect different V_H gene segments depend upon comparison with the known DNA sequences of relatively few variable gene segments. Almost all of these sequences come from V_H subgroups II and III and a large proportion are members of the gene families involved in binding the NP and PC haptens. It is not certain how well these sequences represent the total germline V_H gene repertoire. To increase the probability of detecting V_H expression, we constructed cDNA libraries from three T-cell hybridomas responding to presumably dissimilar antigens. Since each of these cells maintained antigen-specific function, if V_H genes encode the T-cell antigen-binding receptor, each should synthesize an RNA

containing a V_H gene segment. Because the hybrids are the product of two (395A4.4) or more parental T cells (59, 60), they might be expected to express two or more V_H genes if, as in B-cell hybrids, V_H gene segments are transcribed from several chromosome 12 homologues. In addition, it has been suggested that even a single diploid T cell may synthesize more than one V_H RNA (61, 62). Thus, if T cells use the entire V_H gene repertoire, we believe there would have been a V_H segment sufficiently homologous to have been detected by one of our two screening methods. Although it remains formally possible that T cells express selectively a portion of the B-cell V_H gene repertoire containing sequences only distantly related to our probes, no such V_H genes have been characterized.

We have presented strong evidence in favor of the proposition that the helper and suppressor T cells tested do not contain RNA with V_H gene segments. This negative conclusion is not, however, completely compelling and two major objections concerning the detection limit and the range of our library screening, have been discussed. A number of unlikely possibilities, including selection against cloning the V_H-containing sequences or a V_H mRNA that is present mostly in the poly(A)$^-$ fraction, have also not been eliminated. However, using different methods, another laboratory has reported that T lymphocytes do not transcribe any V_H gene segments (63).

Many of the genes which have dominated our thinking about the immune response including β_2-microglobulin and the class I and class II products of the MHC, show clear homology to immunoglobulin genes (64-68). The T cell and B cell antigen-binding receptors presumably have somewhat homologous functions. We would be surprised, therefore, if the genes encoding the T-cell antigen receptor were to have no homology to immunoglobulin genes. Since multigene families are known to duplicate and diverge (69), it is possible that gene families important for B- and T-cell antigen recognition diverged prior to or relatively early in vertebrate

evolution. Attempts to clone T-cell receptor genes using V_H probes might therefore not be feasible, somewhat analogous to attempting to clone V_κ gene segments using V_λ probes. At this point, we can only speculate on the selective forces which may have resulted in separate V gene families expressed in B and T cells.

Summary

We attempted to determine if T cells express any V_H gene segments. cDNA libraries were constructed from one suppressor and two helper T-cell hybridomas. Both the library construction and screening were designed to maximize detection of a wide range of V_H gene segments. One screening method should detect about half of the sequenced V_H genes, while the second should detect most of these genes. The probability of detecting a V_H gene homologous to the probes and present at 10 copies per cell was 77% for one helper cell cDNA library, 88% for the second helper cell library, and greater than 99% for the suppressor cell library. No cDNA clones with V_H gene segments were detected. From this result, we conclude that V_H gene segments are not likely to encode the antigen-specific receptor in the cells we tested.

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TABLE I

T-Cell Hybrids Used to Construct cDNA Libraries

Hybridoma	Normal T-Cell Parent	Fusion Partner	Antigen Specificity	Functional Class	Reference
AODK 10.4	KLH immune B10.D2	AO40.10AG1	KLH + $I\text{-}A^d$	Helper	60
AODH 7.1	HGG immune DBA/2	AO40.10AG1	HGG + $I\text{-}E^d$	Helper	60
395A4.4	GT immune B10.S	BW5147	GT and GAT	Suppressor	59

TABLE II

Homology of Murine V_H Gene Segments to the Hybridization Probes

Cloned V_H Gene Segment	Derivation of Clone[a]	Homology of Cloned V_H Gene Segments to the Hybridization Probes			Comments	Reference
		Synthetic Undecamers[b]	S107V1[c]	BCL1[c]		
V_HT15(V1)	G	11/11	100	56	T15 gene family[e]	39,70
V11	G	11/11	90	59	T15 gene family	70
V13	G	11/11	86	56	T15 gene family	70
S107V1	R	11/11	100	56	T15 gene family	39
M603	R	11/11	98	56	T15 gene family	39
MOPC167	R	10/11	96	56	T15 gene family	71
V14A	G	10/11	75	55	-	S. Crews, unpublished
V14B	G	10/11	73	54	-	S. Crews, unpublished
V_H76	R	10/11	74	55	-	72
V102	G	11/11	58	77	S43 gene family	73
V23	G	10/11	58	78	S43 gene family	73
V3	G	11/11	59	76	S43 gene family	73
V186-1	G	9/11	58	76	S43 gene family	73
V186-2	G	10/11	58	76	S43 gene family	73
S43	R	10/11	56	74	S43 gene family	73
B1-8	R	10/11	58	76	S43 gene family	73

TABLE II (continued)

pCH105	G	10/11	60	78	MPC11 gene family	74
pCH108A	G	11/11	59	82	MPC11 gene family	75
pCH108B	G	11/11	57	80	MPC11 gene family	75
MPC11	R	10/11	59	74	MPC11 gene family	76
V_H101	G	10/11	59	57	-	77
V_H101	R	11/11	60	57	Related to V_H101-G	77
PJ14	G	11/11	57	56		78
M141	R	10/11	57	57	Related to PJ14	78
V_H441	G	11/11	71	57		79
UPC10	R	10/11	70	57	Related to V_H441	80
BCL1	R	8/11	56	100	-	40
J558	R	11/11	-[d]	-[d]	-	Unpublished
MOPC21	R	11/11	71	58	-	73; this paper
V_HB2	R	11/11	54	74	-	81
V_HB49	R	11/11	56	50	-	81
V_HGAT	R	10/11	57	71	-	25
93G7	R	10/11	56	78	-	82
	R	11/11	58	73	-	M. Fougereau, unpublished

[a] G = germline V_H gene segment; R = rearranged V_H gene segment. Five gene segments considered to be pseudogenes have not been included in this compilation.

[b] Synthetic oligonucleotides we synthesized are: 5' GCACAGTA$^{A}/_{G}$TA 3'. The degree of homology is

TABLE II (continued)

expressed as a fraction, the denominator being the length of the oligonucleotides (11) and the numerator is the maximal number of residues homologous to either undecamer.

[c]Percent homology of V_H gene segments to the S107V1 and BCL1 probes. Gaps were introduced where appropriate to compensate for the different lengths of the hypervariable regions. The complete amino acid sequence of the protein produced by the S107 myeloma and the nucleotide sequence of the germline gene (V1) encoding this protein are both available. However, the nucleotide sequence of the rearranged S107V1 cDNA we used as a probe is not complete. Based on the protein sequence we have assumed the rearranged and germline genes are identical, although the possibility of a few silent nucleotide substitutions has not been ruled out.

[d]Insufficient nucleotide sequence data is available.

[e]Gene family denotes a set of closely related sequences. Some members of the T15 family are involved in the response to phosphorylcholine. Some members of the S43 family are involved in the response to NP [4-hydroxy-3-nitrophenyl)acetyl].

TABLE III

Colony Hybridization Results

cDNA Library	Synthetic Oligonucleotides			J_H-Primed Spleen DNA			S107 V_H and BCL1 V_H		
	Colonies Hybridized	Positive Colonies	Frequency	Colonies Hybridized	Positive Colonies	Frequency	Colonies Hybridized	Positive Colonies	Frequency
AODK 10.4	140,000	7	1/20,000	26,000	55	1/500	140,000	0	-
AODH 7.1	200,000	4	1/50,000	18,000	10	1/1800	200,000	0	-
395A4.4	920,000	43	1/21,000	200,000	145	1/1400	920,000	0	-

TABLE IV

Probability of Cloning a Rare mRNA from the T-Cell cDNA Libraries

Hybridoma	pg RNA per Cell (Yield)[a]	pg RNA per Cell (Total)[b]	pg Poly(A)$^+$ RNA per Cell (Yield)[a]	N (Colonies)[c]	1 Copy/Cell		10 Copies/Cell		15 Copies/Cell	
					f[d]	P[e]	f	P	f	P
AODK 10.4	5.8	9.3	.15	105,000	1/715,000	.14	1/71,500	.77	1/46,500	.90
AODH 7.1	3.3	5.0	.05	150,000	1/715,000	.19	1/71,500	.88	1/46,500	.96
395A4.4	3.1	4.8	.13	690,000	1/715,000	.62	1/71,500	<.99	1/46,500	<.99

[a]RNA yield estimated from absorbance at 260 nm.

[b]Calculated by multiplying the yield of RNA by the recovery of a small amount of ^{3}H-labeled sea urchin RNA added to the preparation. Recovery was at least 60% in each case.

[c]N is the number of colonies in the library hybridized; N has been corrected on the assumption that 25% of the RNA is ribosomal or other nonpolyadenylated species. This fraction was estimated following gel electrophoresis of the poly(A)$^+$ RNA.

(continues)

Table IV (continued)

[d]f is the fraction of the mass of total cell poly(A)$^+$ RNA present in a given sequence. We assumed each cell contains 0.3 pg of poly(A)$^+$ RNA. We considered a sequence of 800 nucleotides, the average length of the cDNA clones, present at either 1, 10, or 15 copies per cell.

[e]Probability of cloning a gene calculated from the formula.

$P = 1 - (1-f)^N$; assumes the probability of detecting a clone is a function of its abundance in the RNA population. See discussion for details.

FIG. 1. Synthetic oligonucleotide probes. The conserved oligonucleotide sequences found in immunoglobulin V gene segments, the amino acids these sequences encode, and the complementary undecamers we synthesized are shown.

Protein Sequence:		Tyr	Tyr	Cys	Ala		Tyr	Tyr	Cys	Ala	
Conserved DNA Sequences:	5'	TAT	TAC	TGT	GC	3'	5'	TAC	TAC	TGT	CG	3'
Synthesized Complementary Oligonucleotides:	3'	ATA	ATG	ACA	CG	5'	3'	ATG	ATG	ACA	CG	5'

Figure 1

FIG. 2. Synthesis of J_H-primed cDNA from spleen RNA. Coding sequences on the immunoglobulin heavy chain messenger RNAs are indicated as follows: C, constant region; J, joining gene segment; V_a and V_b, two different V_H gene segments. The J_H primer is indicated by the thick horizontal line. The short vertical lines represent hydrogen-bonded base pairs and the X's represent radioactive nucleotides incorporated into cDNA.

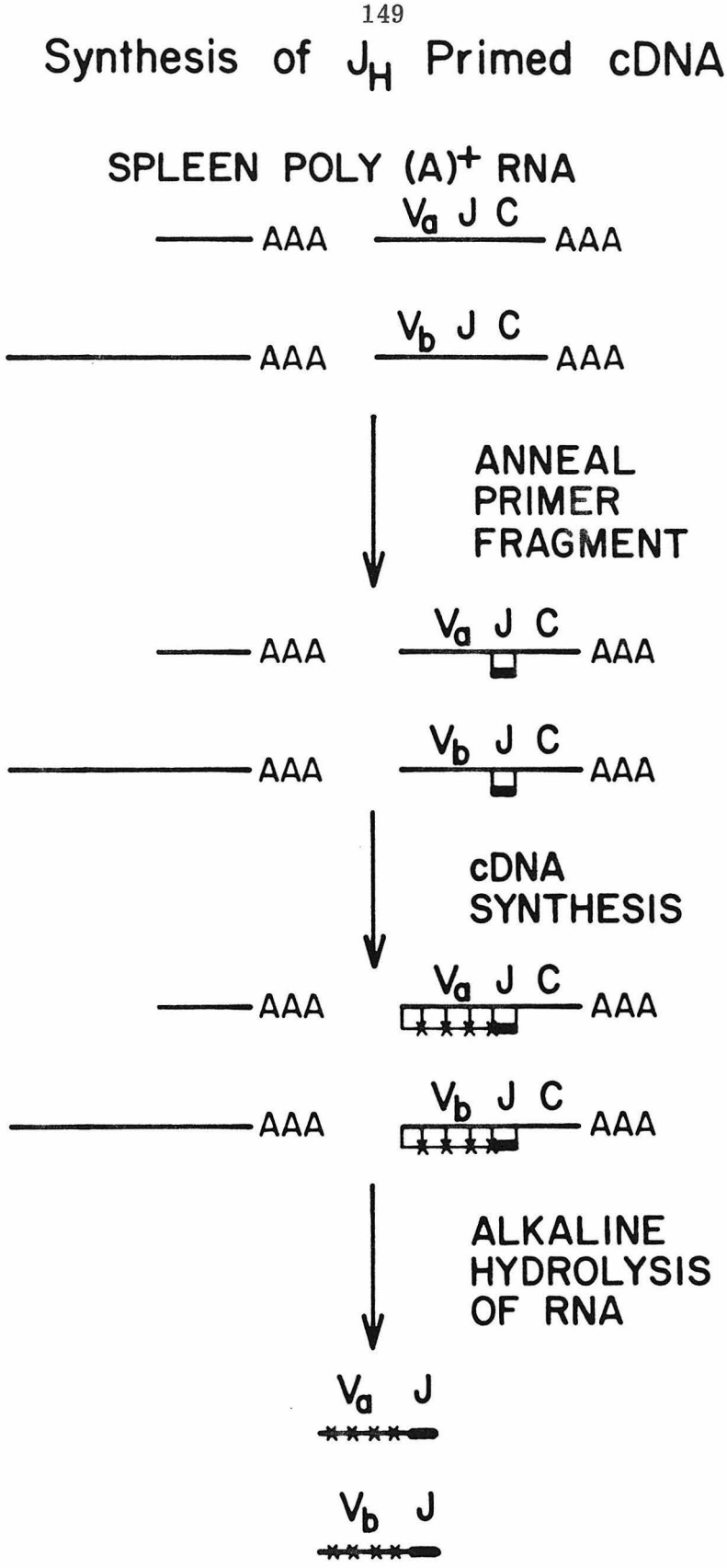
Synthesis of J_H Primed cDNA
SPLEEN POLY (A)+ RNA
AAA
V_a J C
AAA
V_b J C
ANNEAL PRIMER FRAGMENT
cDNA SYNTHESIS
ALKALINE HYDROLYSIS OF RNA
V_a J
V_b J

Figure 2

FIG. 3. J_H primer fragments. The sequence of the primer fragments, used to generate the spleen cDNa probe, is shown (78). Digestion of a 2 kb restriction fragment containing the four J_H gene segments generates four separate fragments containing the J_H coding sequences and about 30 fragments from sequences flanking the J_H gene segments. Only the J_H coding sequences are shown. Restriction enzyme sites are indicated with vertical lines and the primer fragments generated are enclosed in rectangles.

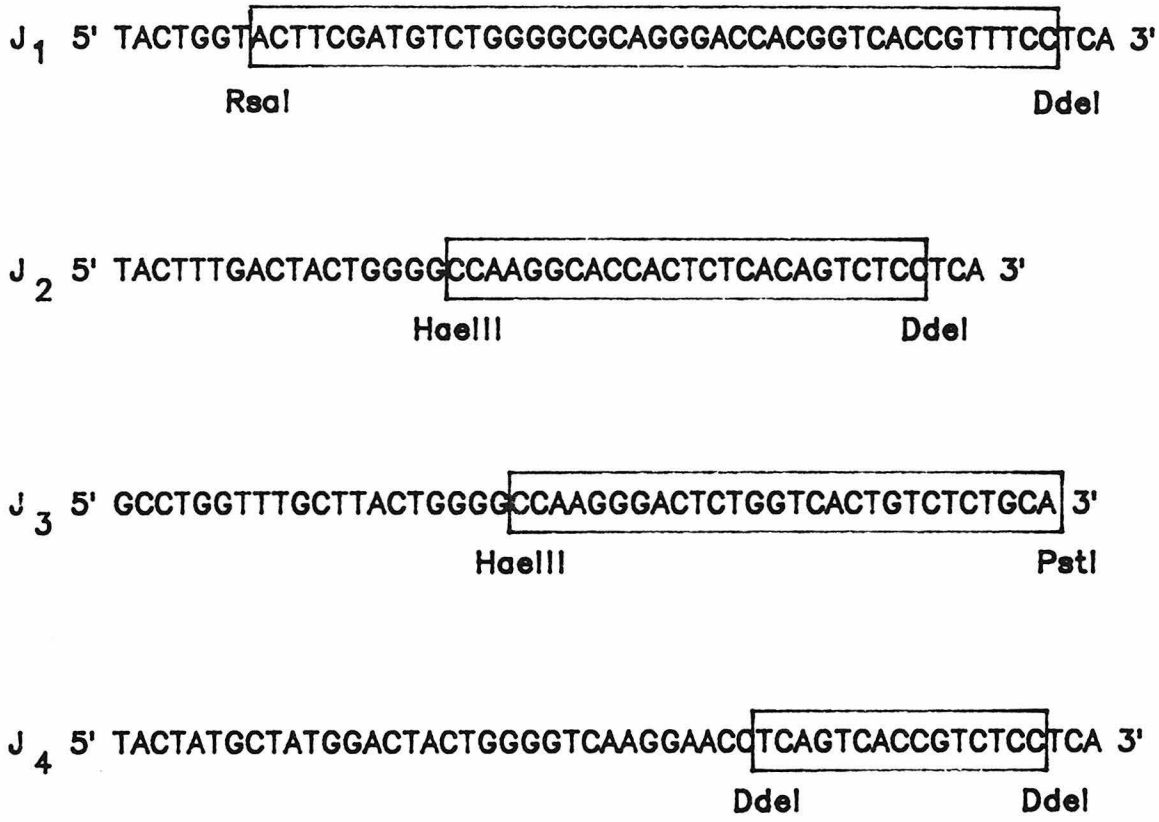
J1 5' TACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTTTCCTCA 3'
RsaI
DdeI
J2 5' TACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA 3'
HaeIII
DdeI
J3 5' GCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA 3'
HaeIII
PstI
J4 5' TACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA 3'
DdeI
DdeI

Figure 3

FIG. 4. Positive control hybridizations to cDNA colonies containing a cloned V_H gene segment. A circular nitrocellulose filter containing DNA from several thousand identical pF9V21 (MOPC21) V_H cDNA clones was cut into sections. Hybridization conditions are described in the Materials and Methods section. The hybridization probes were: (a) Radiolabeled synthetic oligonucleotides. The filter was exposed to film for 12 h. (b) Radiolabeled J_H-primed spleen cDNA. The filter was exposed for 24 h. (c) pVH_3 plasmid containing the BCL1 V_H gene. The filter was exposed for 10 h. The same section of filter was hybridized to the synthetic oligonucleotides, washed to remove the probe, and was then hybridized with the J_H-primed spleen cDNA.

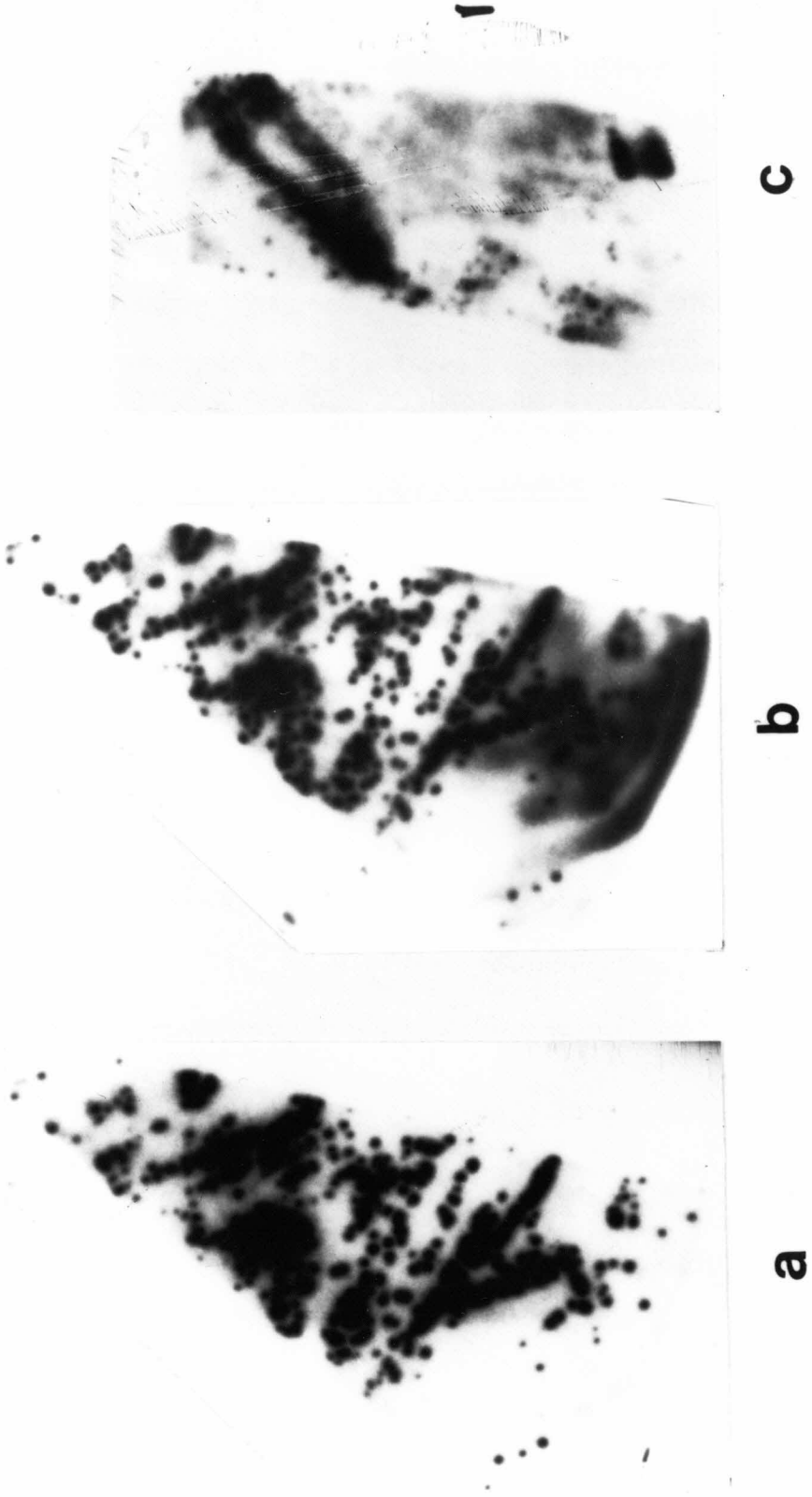

Figure 4

FIG. 5. Hybridization of specifically primed spleen cDNA to clones that contain V_H genes. (A) Agarose gel stained with ethidium bromide. 1, BamHI digest of plasmid containing V_HB2 sequence. 2, EcoRI digest of a bacteriophage λ clone containing two V gene segments, V14A and V14B. These V genes should be located on separate restriction fragments 5.0 and 2.0 kilobases long. 3, BamHI digest of a bacteriophage λ clone containing the T15 V_H gene segment. The T15 V_H sequence is located on a 2.4 kilobase restriction fragment. (B) Southern blot of DNA in (A) hybridized to the specifically primed spleen cDNA. Three fragments containing V gene segments gave detectable hybridization. In lane 2, the 2.0 kilobase fragnnet containing a V gene segment did not hybridize. The higher molecular weight fragment in lane 3 may be uncut DNA. Migration distances of the molecular weight markers are shown. kb, kilobase.

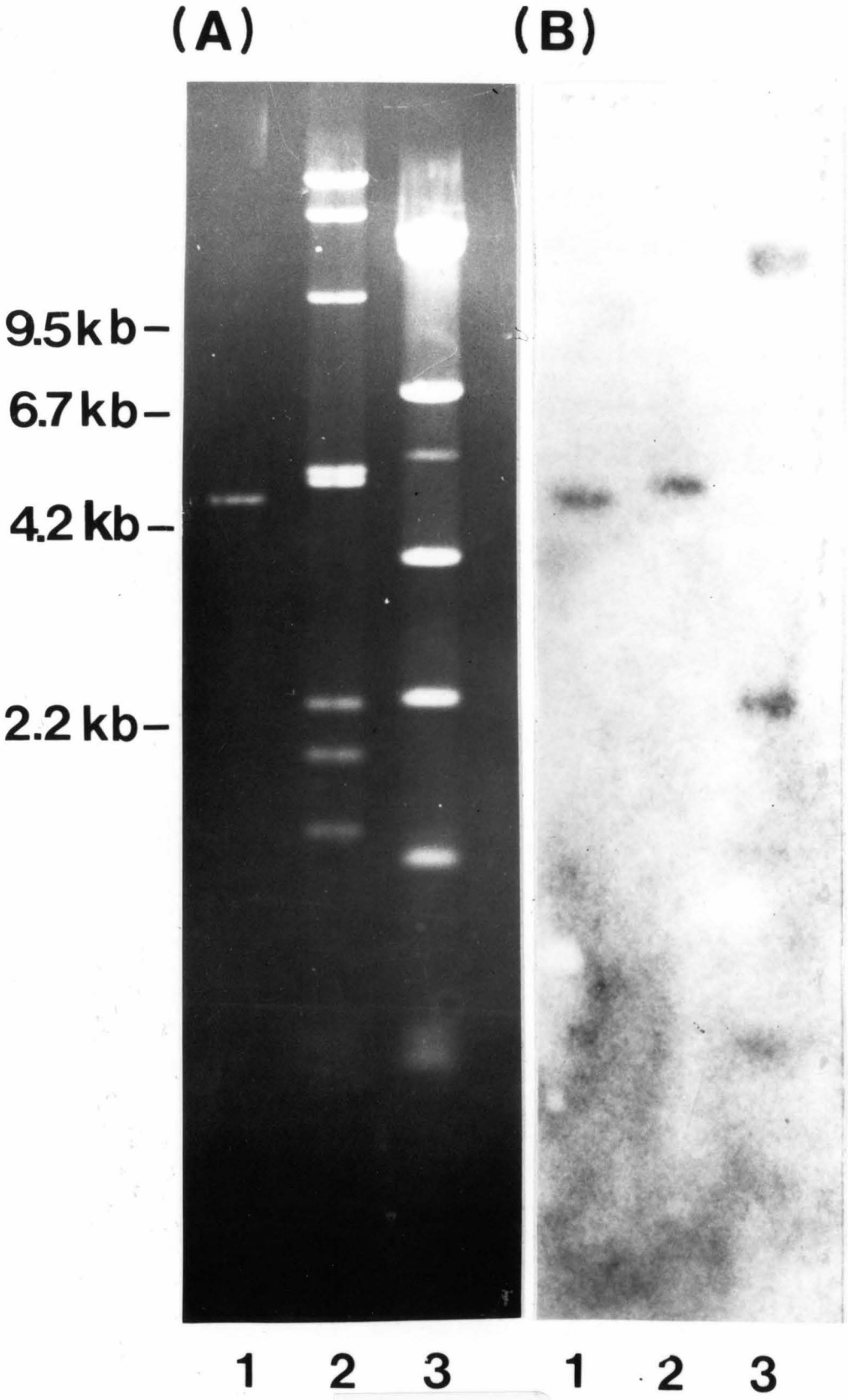

Figure 5

FIG. 6. Nucleotide sequence of a V_H cDNA clone (F9V21) detected with the synthetic oligonucleotides and J_H-primed spleen cDNA. The predicted amino acid sequence is shown above the DNA sequence. The sequence agrees with one previously reported for the MOPC21 V_H gene (73), except for the 209th nucleotide which was cytidine instead of adenosine. This difference is silent with respect to the predicted amino acid sequence. As noted previously, there are six discrepancies between the published amino acid sequence for MOPC21 and the nucleotide sequence (45, 73). The F9V21 cDNA clone begins in the middle of the codon for the second amino acid, and contains the entire V_H, D, and J_H4 gene segments. A portion of the D and the entire J_H gene segment are not shown. The 11-nucleotide sequence complementary to one of the synthetic nucleotides is underscored.


Q L V E S G G G L V Q P G G S R K L S C A A S G F
1 TGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCGGAAACTCTCCTGTGCAGCCTCTGGATTC 77
T F S S F G M H W V R Q A P E K G L E W V A Y I S
78 ACTTTCAGTAGCTTTGGAATGCACTGGGTTCGTCAGGCTCCAGAGAAGGGGCTGGAGTGGGTCGCATACATTAGT 152
S G S S T L H Y A D T V K G R F T I S R D N P K N
153 AGTGGCAGTAGTACCCTCCACTATGCAGACACAGTGAAGGGCCGATTCACCATCTCCAGAGACAATCCCAAGAAC 227
T L F L Q M T S L R S E D T A M Y Y C A R W G N Y
228 ACCCTGTTCCTGCAAATGACCAGTCTAAGGTCTGAGGACACGGCCATGTATTACTGTGCAAGATGGGGTAACTAC 302

Figure 6

FIG. 7. Hybridization of the synthetic oligonucleotides and J_H-primed spleen cDNA to T-cell cDNA colonies. A single circular filter containing several thousand colonies from the 395A4.4 suppressor T-cell cDNA library is shown. (A) Hybridization with the synthetic oligonucleotide. The arrows indicate three colonies to which hybridization of the radiolabeled synthetic oligonucleotides was detected in a 72-h exposure. A duplicate filter gave the same pattern of oligonucleotide-positive colonies. (B) Hybridization with the J_H-primed spleen cDNA. After incubation of the filter at 50°C to remove the hybridized oligonucleotides, the same filter as shown in (A) was hybridized with the radiolabeled J_H-primed spleen cDNA. The signal from positive colonies varies and some nonspecific background is present. The filter was exposed for 48 h. The arrows mark the position of the three oligonucleotide-positive colonies. None of the colonies hybridized with both probes.

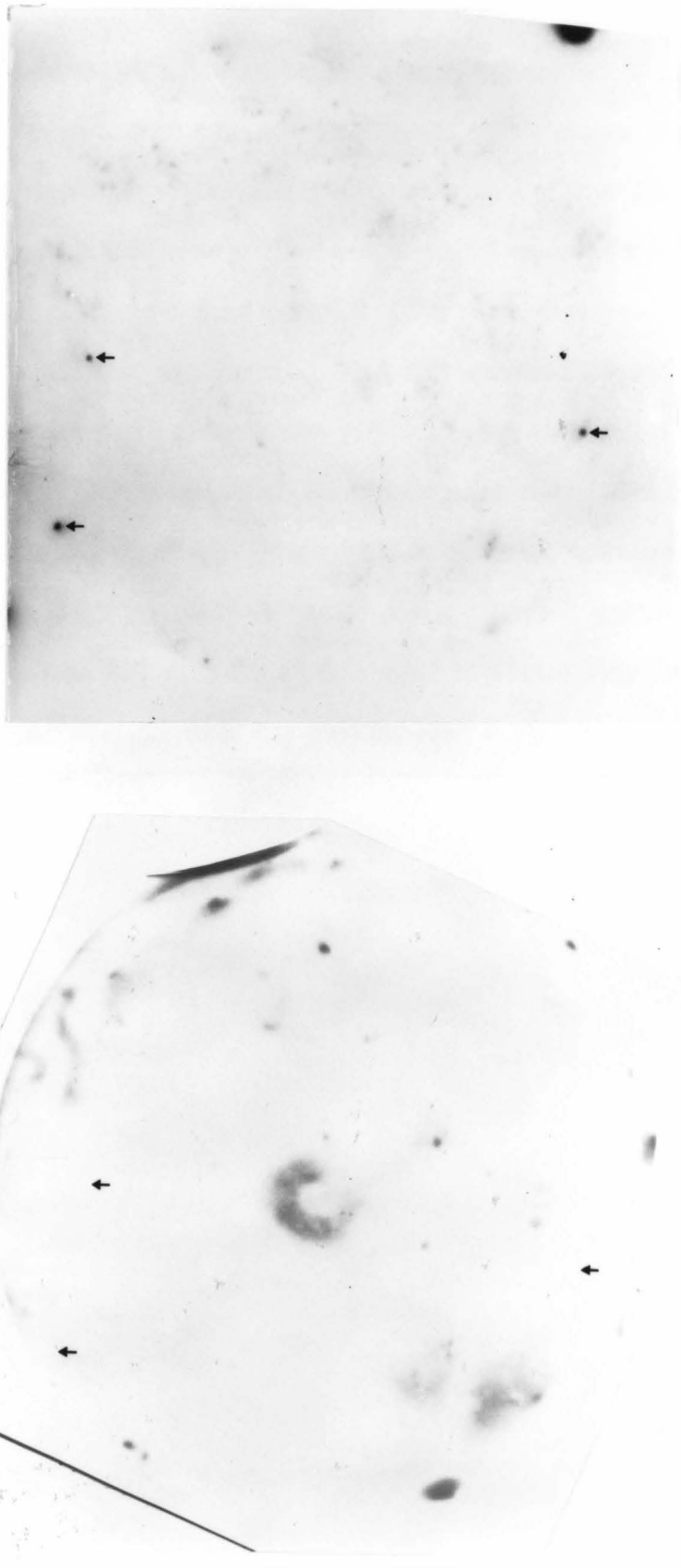

Figure 7

FIG. 8. Southern blots of some oligonucleotide-positive T-cell cDNA colonies. Plasmid DNA was prepared from several T-cell cDNA clones which hybridized with the synthetic oligonucleotides. Restriction mapping of the plasmids indicated they contain an average of 800 base pairs of mouse cDNA inserted into the pBR322 cloning vector (4.36 kb). The purified plasmid DNA was digested with the restriction enzymes Eco RI and Pvu II. Digested DNA was electrophoresed on 1% agarose gels and blotted onto nitrocellulose sheets. Migration distances of some molecular weight markers, and their lengths in kilobases, are indicated. Lanes 1-3 and 6-9 contain DNA from separate oligonucleotide-positive colonies isolated from the 395A4.4 library. Lane 4 contains pBR322 vector DNA. Lane 5 contains the MOPC21 V_H cDNA. (a) Hybridization with the radiolabeled synthetic oligonucleotides. Exposure for 3 h. (b) Hybridization with the radiolabeled J_H-primed cDNA. The filter was exposed for 24 h.

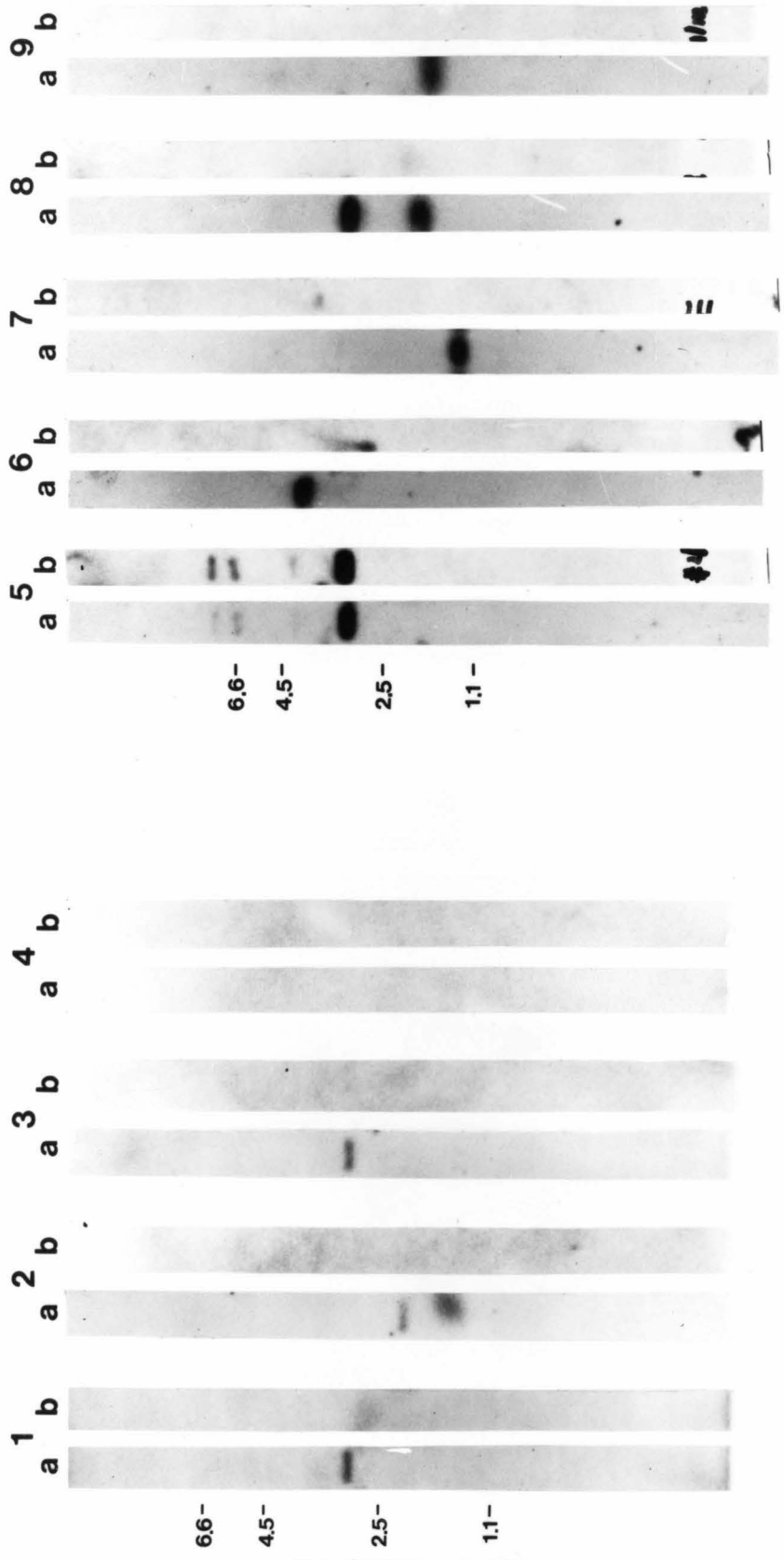

Figure 8

Chapter 6

RNA TRANSCRIPTS FOR I-J POLYPEPTIDES ARE APPARENTLY NOT ENCODED BETWEEN THE I-A AND I-E SUBREGIONS OF THE MURINE MAJOR HISTOCOMPATIBILITY COMPLEX

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Classification:

RNA transcripts for I-J polypeptides are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex

(Northern blots/T cell hybridomas/suppressor cells/I region genetics)

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Abbreviations: kb, kilobase(s); MHC, major histocompatibility complex.

ABSTRACT The I-J subregion of the mouse major histocompatibility complex has been reported to encode polypeptides expressed by suppressor T cells. Previously, we obtained cosmid clones from mouse sperm DNA which contain all of the sequences between the I-A and I-E subregions, where I-J has been mapped genetically. However, hybridization of these sequences to RNA prepared from several I-J positive, suppressor T cell hybridomas did not reveal the presence of a transcript. In addition, no rearrangements in this DNA were detected in the suppressor T cells that we have analyzed. Our results indicate that the I-J polypeptides are not encoded between the I-A and I-E subregions of the major histocompatibility complex. We discuss several hypotheses concerning the possible location and expression of I-J genes.

The I region of the murine major histocompatibility complex (MHC) encodes polymorphic cell-surface molecules which are involved in lymphocyte interactions (1, 2). The I region has been divided by analysis of recombinant inbred mice into five subregions arranged in the order: I-A, I-B, I-J, I-E, and I-C (Fig. 1A) (3). The I-A and I-E subregions code for class II molecules or Ia antigens which are found predominantly on the surfaces of B cells and macrophages (4, 5). These molecules are composed of two polypeptides, an α chain of approximately 32,000 daltons, and a β chain of approximately 28,000 daltons (6). In order to mount an immune response, some T lymphocytes, including helper or inducer T cells, must recognize syngeneic class II molecules in addition to the stimulating antigen (7, 8). The other subregions, I-B, I-J, and I-C, are less well characterized. For example, while the I-B subregion may affect the level of the immune response to certain antigens, it does not encode serologically detectable molecules and its existence is controversial (9, 10). On the other hand, numerous alloantisera and monoclonal antibodiies have been raised against lymphocytes from strains which differ only in the I-J subregion (11-14). The antigens recognized by these antibodies have a unique tissue distribution; they are found predominantly on the surface of suppressor T cells and on soluble factors with suppressive activity secreted by these cells (11-16). In addition, there are some reports of I-J positive subsets of helper T lymphocytes (17) and macrophages (18). Interest in the I-J subregion and its gene products has been heightened by two findings. First, I-J-encoded molecules are associated with or are actually part of the antigen-binding polypeptides made by suppressor T cells (19-21). Thus, a biochemical characterization of I-J molecules may help to define the T-cell antigen-binding receptor. Second, in some cases I-J-encoded molecules appear to regulate interactions between T lymphocytes (22). Thus, they may be models for proteins which mediate cell-cell communication.

There are several reports indicating that the I-J specificity is present on

proteins with a molecular weight of approximately 25,000 daltons (20, 21, 23). In addition, because the I-J serological determinant can be found on proteins translated in vitro by a rabbit reticulocyte system, the antigenic determinant is presumed to be a polypeptide rather than a carbohydrate structure (23). Little more is known, however, about the structure of I-J-encoded molecules.

Recently we have been able to define a 2.0 kilobase (kb) DNA sequence which should contain the gene(s) encoding the I-J serologic specificity (24, 25). First, using a BALB/c mouse ($H\text{-}2^d$) cosmid library and two human cDNA probes, we have isolated 200 kilobases (kb) of contiguous DNA sequence located in the I region. The coding sequences for the E_β chain encoded in the I-A subregion and E_α chain encoded in the I-E subregion were found to be separated by only 33 kb of DNA (Figs. 1B, 1C) (24). Next, to define more precisely the boundaries of the I-A and I-E subregions, we identified polymorphic restriction enzyme sites in the I region and tested genomic DNA from parental and I region recombinant strains for the presence of these polymorphic sites (24, 25). This analysis permitted us to correlate the genetic map of the I region with the molecular map of the cosmid cluster. We discovered that the boundaries of the I-A and I-E subregions, which define I-J, are less than 2.0 kb apart (Fig. 1D).*

There are two reasons why this region seems too small to encode the I-J polypeptides. First, the 2.0-kb sequence is actually contained within the E_β gene and includes part of the intron between the first (β1) and second (β2) major exons and probably all of the second major exon (β2) of the E_β gene (Figs. 1D, 1E). Second, distinct I-J-encoded specificities have been detected on different T-cell subpopulations or cloned T cells, suggesting that the I-J subregion could in fact contain several genes (26-28). To resolve this problem, we have proposed several models for the location and expression of the I-J gene(s) (Table 1) (24). One set of models (1a, 1b, 1c, and 1d) proposes that all or part of the I-J gene is located between the I-A

and I-E subregions. These models have been excluded in the experiments reported below. A second set of models (2a, 2b, and 2c), which propose that the I-J-coding sequence is located elsewhere, are considered in the discussion.

MATERIALS AND METHODS

Cells. T suppressor hybridomas are described in Table 2. Three T helper hybridomas were obtained from the laboratory of Drs. John Kappler and Philippa Marrack, National Jewish Hospital, Denver, Colorado. These cells are: AODK 10.4, which is specific for keyhole limpet hemocyanin and $I\text{-}A^d$ (31), AODH 7.1, which is specific for human gammaglobulin and $I\text{-}E^d$ (31) and DO 11.10 which is specific for chicken ovalbumin and $I\text{-}A^d$ (32).

Northern Blots. $Poly(A)^+$ RNA was prepared (33,34), electrophoresed in denaturing gels (35) and transferred to nitrocellulose (36) as previously described. DNA probes were nick-translated (38) to a specific activity of 8×10^8 cpm/μg and were hybridized to Northern blots at a concentration of 2×10^5 cpm/ml in the presence of 50% (vol/vol) formamide (37) and 10% (wt/vol) dextran sulfate. To reduce background caused by the hybridization of repeat nucleotide sequences in the probes, both the prehybridization and hybridization solutiosn contained 25 μg/ml of denatured BALB/c liver DNA. The filters were washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 58°C and exposed to Kodak XAR-5 film in the presence of an intensifying screen for 4 days.

Southern Blots. High molecular weight DNa was prepared from T cell hybridomas, T lymphoma BW5147, and from mouse liver according to the method of Blin and Stafford (39). DNA was electrophoresed, blotted to nitrocellulose (40) and then was hybridized with the radiolabeled DNA probes as described above.

Hybridization Probes. Hybridization probes derived from the I region included the cosmids depicted in Fig. 1B and the sequences diagrammed in Fig. 1E. 8.48 and 8.47 are 7.2 and 4.8 kb HindIII restriction fragments from cosmid 8.4, subcloned into

pBR325 by standard methods (41, 42). Probe 3, a 2 kb EcoRI restriction fragment derived from cosmid 24.2 and probe 4, a 500 base pair Sau3A fragment purified from cosmid 39.1, have been described previously (24). pH-2IIa is a 688 base pair PstI-PvuII subclone from a cDNA clone encoding an H-2^d transplantation antigen (43).

RESULTS

Transcripts Homologous to the Presumed I-J Subregion Are Not Detectable in Suppressor T Cells. To identify I-J transcripts, we analyzed 13 suppressor T-cell hybridomas for the presence of RNA molecules which hybridize to cloned DNA probes which include all the sequences between the I-A and I-E subregions. Some properties of these suppressor cells are summarized in Table 2. Nine of the hybridomas were tested either with anti-I-J alloantisera, monoclonal antibody or both and in each case the cells were found to be I-J positive. Because extensive sequence polymorphism might greatly reduce hybridization of probe derived from the H-2^d haplotype to other I-J alleles, we tested one hybridoma generated from BALB/c lymphocytes, the same inbred strain from which the cosmid clone bank had been prepared. Northern blots of poly(A)$^+$ RNA from each of the T cells was hybridized with a mixture of the ^{32}P-labeled 8.48 and 8.47 subclones (Fig. 1D). Each RNA preparation was tested on at least two Northern blots, and in no case were transcripts detected in the suppressor T-cell RNA. Results obtained with nine of the suppressor cells are presented in Fig. 2. Since MHC-encoded transplantation antigens are expressed by most cell types, rehybridization of the same Northern blots with pH-2IIa, a probe encoding an H-2^d transplantation antigen, provided a positive control for the presence of intact RNA (Fig. 2B). Although the hybridomas transcribe varying amounts of RNA homologous to this probe, in 12 cases a distinct 1.9-kb band was detected. One hybridoma, 368B1.5, transcribes very low amounts of class I sequence which can be detected only on very long exposures (44). Additional control hybridizations were carried out using the I region subclones and various amounts of

spleen poly(A)$^+$ RNA. We could detect hybridization of the E_β coding sequence in these subclones to 0.1-0.3 μg of spleen RNA. . By comparing the E_β hybridization signal to the signal obtained with a kappa light chain constant region probe hybridized to a known amount of purified S107 myeloma κ chain RNA, we estimate that the E_β RNA represents approximately 0.03 percent of the spleen poly(A)$^+$ RNA (unpublished data). Similar estimates for the abundance of class II mRNA have been reported by other laboratories (45-47). Figure 2 demonstrates that we could detect an RNA species which is 0.03 percent of 0.3 μg spleen RNA tested. Thus we should have been able to detect sequences present at less than one part in 10^5 contained in the 10 μg of T cell RNA analyzed. Since the E_β RNA is about 1300 nucleotides long and T-cell hybridomas generally contain 0.2 to 0.3 pg of poly(A)$^+$ RNA per cell (48), our detection limit is roughly 3-4 copies per cell.

Transcripts Homologous to 170 kb of Cloned I Region DNA Are Not Detectable in Five T Cell Hybridomas. To determine whether any cloned I region sequences are transcribed in T lymphocytes, we hybridized the six full-length cosmid DNA clones indicated in Fig. 1B singly, or in pairs, to poly(A)$^+$ RNA from the 368B1.5 and 372B3.5 suppressor hybridomas and to poly RNA(A)$^+$ RNA from the AODH 7.1, AODK 10.4, and DO 11.10 helper T-cell hybridomas. No discrete transcritps were found in any of the T-cell hybridomas (Fig. 3 and unpublished data). Occasionally, however, we observed a faint smear of hybridization to high molecular weight RNA (Fig. 3). We believe this is caused by hybridization of repeat sequences in the cosmid probes to poly(A)$^+$ nuclear RNA, since similar smears were observed in liver RNA as well (unpublished data). Therefore, we conclude that no I-J exons are located on 170 kb of cloned I region sequence. As described above, a positive control was obtained in each case by rehybridizing the RNA with a probe encoding an H-2^d transplantation antigen (unpublished data). As an additional control, we found that hybridization of cosmids 41.1, 24.2, 8.4, and 17.2 detected a 1.3-kb transcript in 0.3 μg of poly(A)$^+$ RNA from

BALB/c spleen, while cosmids 21.1 and 7.1 did not detect any transcripts using 3 μg of RNA (Fig. 3 and unpublished data). These results are consistent with the map of I region coding sequences depicted in Fig. 1C.

The I-J Gene Is Not Formed by Rearrangement of DNA Sequences Between the I-A and I-E Subregions. We determined whether a DNA rearrangement in suppressor T cells might form an I-J gene. High molecular weight DNA that was prepared from four T-cell hybridomas. was digested with one of several restriction enzymes, and was hybridized with some of the cloned probes indicated in Fig. 1D. No DNA rearrangements were observed on any of these Southern blots. Figure 4 shows some cases in which the enzyme digest allowed us to distinguish between those restriction fragments derived from the normal T-cell parent and those from the BW5147 thymoma. Because the probes contain repeated sequences, sometimes a series of faint bands not predicted by the restriction map of the cosmid clones were also visible (Fig. 4, lanes 10-12).

DISCUSSION

Using a series of cosmids, we have previously determined that the boundaries of the I-A and I-E subregions are separated by only 2.0 kb of DNA (24, 25). This analysis confines the I-J subregion to a relatively short sequence which is actually located within the E_β gene (Fig. 1). A number of experimental results have convinced us that the BALB/c cosmid DNA map is an accurate representation of the germline sequences found between the E_β and E_α genes. First, we have obtained seven different overlapping cosmid clones containing all or part of the DNA between E_β and E_α (24). This eliminates the possibility that the map fuses noncontiguous sequences and thereby misses a gene. Second, extensive comparisons of the restriction enzyme maps between BALB/c mice and other strains indicate that the BALB/c genomic DNA contains neither a deletion nor an inversion breakpoint between the E_β and E_α genes

(24, 25). Finally, we have obtained cosmid DNA clones from the AKR inbred strain which has a MHC haplotype ($H\text{-}2^k$) different from BALB/c. In this strain, the E_β and E_α genes are also separated by approximately 33 kb (unpublished data).

We have found no evidence for an I-J coding sequence located between the I-A and I-E subregions. If some I-J exons were present between the I-A and I-E subregions there could be two reasons for failing to detect them in the poly(A)$^+$ RNA: 1) When cloned, suppressor hybridomas are grown in vitro, the I-J serologic specificities can often be detected on only a fraction of the cells (26, 27). Thus, if many cells in a population do not express an I-J polypeptide, the average concentration of I-J transcripts could be extremely low. Although the cells used for RNA purification were tested for antigen-specific function, we cannot state what fraction displayed I-J serologic determinants at the time of harvest. However, it seems improbable that such a low level of I-J transcription, with little quantitative variation, would be maintained in each of 13 suppressor T cells grown in two different laboratories and shown to express high levels of antigen-specific suppressor activity. 2) It is possible that only a single exon, encoding a small portion of the I-J gene product, actually is located between the I-A and I-E subregions. A polymorphic sequence, which perhaps could be as small as the immunoglobulin D gene segment, would not have been detected on an RNA blot. Such a short segment could be co-expressed with other exons located outside the 2.0-kb sequence via RNA splicing. However, we have hybridized cosmid probes spanning most of the 200 kb of cloned I region DNA to Northern blots of poly(A)$^+$ RNA from two suppressor T cells and have not detected any complementary sequences (Fig. 3 and unpublished data). Therefore any short exon between the I-A and I-E subregions would have to be present on an extremely large primary transcript originating outside the sequences we have cloned. The minimum length of such a transcript, assuming that the I-J exon(s) are located at one of the ends of the messenger RNA, would be appropriately 60 kb. Alternatively,

a short exon encoding the polymorphic I-J determinant could be co-expressed with other exons following a DNA rearrangement. However, the Southern blots we have performed exclude this possibility as well (Fig. 4).

We conclude that the genes encoding I-J serologic determinants expressed by suppressor T cells do not map between the I-A and I-E subregions. There are several possibilities which are outlined in Table 1. (a) I-J may be linked to the MHC, but the assigned map position is incorrect. The genetic mapping is based upon the analysis of a few recombinant mice and the reasonable assumption that multiple crossovers are rare. Yet in some regions of the mouse genome, it is known that multiple crossovers are relatively common (49). A recombination hotspot which has been localized within or near to the boundaries of the I-A and I-E subregions (24) may have affected the genetic localization of I-J. This hotspot could be associated with a high frequency of multiple crossovers (negative interference) or some other anomaly. However, we have no data to support this speculation and we do not know of any documented cases of double recombinants involving the well-characterized MHC loci. (b) Each inbred mouse strain may contain several pseudoallelic I-J genes and a regulatory locus which determines the I-J gene expressed. To explain the reported properties of I-J gene products, the regulatory locus must be polymorphic, it must map between the I-A and I-E subregions and it must regulate expression of the pseudoallelic genes in a haplotype-specific manner. There are proven no examples of such a polymorphic regulatory sequence. (c) Because T cells have antigen receptors which recognize either self class II molecules, or a self class II-antigen complex, the I-J serologic determinant actually may be present on the T-cell antigen receptor. The linkage of the I-J specificity to the MHC in laboratory mouse strains could then be explained if polymorphic class II molecules select for expression of anti-self T-cell receptors from a receptor gene pool which does not vary greatly in these inbred strains. The genetic map would then predict that the suppressor T cells have receptors which recognize

determinants encoded by the E_β gene. However, some inbred mouse strains may not express E_β (50), while some strains which express different I-J alleles may have identical E_β genes (51).

In summary, our experiments indicate that I-J exons are not likely to be present between the boundaries of the I-A and I-E subregions. While none of the three alternative models for the location of the I-J gene is entirely satisfactory, the possibility of a high frequency of multiple crossovers leading to an incorrect map for the subregions of the I region order is most attractive, since this hypothesis requires the fewest ad hoc assumptions and special mechanisms to account for the reported data. We have cloned the recombination point between the I-A and I-E subregions in several I region recombinant strains (25). Further characterization of these recombinants as well as the isolation of cloned sequences encoding the I-J polypeptide will finally resolve the paradox of I-J gene location and expression.

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Footnote

*The 2.0-kb sequence is defined on the left side (I-A) by a polymorphic restriction site which distinguishes H-2^b from H-2^d DNA, and on the right side (I-E) by a site which distinguishes H-2^k from H-2^s (25). The localization of I-J to the 2.0-kb sequence therefore assumes that both anti-I-J^b and anti-I-J^k plus anti-I-J^s alloantisera recognize a specificity encoded by the same or else a nearby exon of the I-J gene. If this is not the case, then the I-J gene may be encoded in a somewhat longer sequence. However, it should be noted that the 8.48 and 8.47 Northern blot hybridization probes covered approximately 12 kb of DNA in this region (Fig. 1D).

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Table 1. Models for the location of the I-J gene

1.	The I-J gene is encoded between the I-A and I-E subregions.
	a. The I-J gene is actually the E_β gene. The I-J specificity is formed via special post-translational modification (e.g., glycosylation) of the E_β gene product.
	b. The I-J gene is formed via an alternative RNA splicing pattern which includes some of the E_β exons and some exons unique to I-J.
	c. The I-J gene is transcribed from the DNA strand complementary to that which encodes the E_β gene.
	d. The I-J gene is formed by a DNA rearrangement in suppressor T lymphocytes, which inserts some coding sequences between the I-A and I-E subregions.
2.	The I-J structural gene is not encoded between the I-A and I-E subregions.
	a. Because of the occurrence of multiple recombination events, the map order I-A/I-J/I-E is incorrect.
	b. The I-J subregion contains a control element which regulates the expression of I-J genes encoded elsewhere.
	c. The I-J serologic specificities are present on T-cell receptors for self MHC molecules.

Table 2. Characteristics of suppressor Tcell hybridomas

Number	Hybridoma	T-parental H-2 haplotype*	Antigen specificity+	I-J‡
1	258C4.4	q	GAT/GA	I^q(a); I-J^k(m)
2	342B1.11	s	GAT/GT	I-J^s(a); I-J^k(m)
3	365C6.4	s	GAT	I-J^k(m)
4	367A5.2	k	GT	-
5	368B1.5	d	GT/GAT	I^d(a)
6	301D4.5	f	GT	-
7	301A2.3	f	GT	-
8	469B5.5	a	GT/GAT	I-J^k(a)
9	372B3.5	b	GAT/GT	I-J^b(a,m); I-J^k(m)
10	372D6.5	b	GAT/GT	I-J^b(a)
11	395A4.4	s	GT/GAT	-
12	7C3-13	k	NP	I-J^k(a,m)
13	7F4-30	k	NP	I-J^k(a,m)

*All the hybridomas were formed by fusion of lymphocytes to the HAT-sensitive AKR thymoma BW5147, and they therefore contain H-2^k haplotype DNA contributed by the tumor parent.

+Antigen-binding specificities of T hybridoma suppressor factors. GA is a synthetic polymer of a glutamic $acid^{60}$:$alanine^{40}$; GAT is glutamic $acid^{60}$:$alanine^{30}$:$tyrosine^{10}$; GT is glutamic $acid^{50}$:$tyrosine^{50}$; NP is 4-hydroxy-3-nitrophenyl acetyl. In all cases, the immunogen is listed first followed by any other known specificities.

‡I-J specificities found on the suppressor T cell hybridomas. - = not tested. a = positive with specific alloantisera. m = positive with monoclonal antibody. Suppressor 3 was tested for cell-surface I-J expression only. Suppressors 5, 8, and 10 were tested for the presence of I-J on a soluble suppressor factor. The other positive cells were tested for I-J on both the cell surface and on the

Table 2 (continued)

suppressor factor, and were doubly positive in all cases. For suppressors 2, 3, and 9 a monoclonal anti-I-J^k antibody detected a specificity presumably derived from a BW5147 gene activated post-fusion. The monoclonal anti-I-J^k is believed to cross-react with the I-J^q suppressor factor made by suppressor 1. The alloantisera used to test suppressors 1 and 5 are not specific for I-J alone, but should recognize all I region differences. Further details concerning the serologic testing can be found in the references (14, 27, 29, 30).

Fig. 1. (A) Some of the genetically defined loci in the mouse major histocompatibility complex are indicated. K and D are cell-surface molecules important for graft rejection and target recognition by cytotoxic lymphocytes. S encodes some of the components of the complement cascade. The other loci are described in the text. I-B has been omitted, since it is confined to the same 2.0 kb sequence as I-J, and codes for no serologically detected product. The map is not drawn to scale. (B) I region molecular map. The I subregion boundaries and the length and position of the cosmid hybridization probes are indicated. (C) The locations of the known I region coding sequences are indicated by the rectangles. Intervening sequences are not depicted except the long intron between the first and second external domains of the E_β gene. $E_{\beta 2}$ is not yet well characterized and could be a pseudogene. The E_α and E_β polypeptides associate to form the I-E molecule, while A_β associates with A_α to form the I-A protein. Figs. 1B and 1C are drawn on the same scale. The 19-kb sequence in Fig. 1C demarcated by vertical lines is shown enlarged in Figs. 1d and 1E. (D) The area near the bounaries of the I-A and I-E subregions is depicted. 8.48 and 8.47 were used to analyze T suppressor cells for I-J transcripts, while probes 3 and 4 were used to test these cells for DNA rearrangement. (E) The direction of transcription and the approximate location of the exons of the E_β gene are indicated. $\beta 1$ and $\beta 2$ code for the first and second external domains, while Tm indicates the transmembrane/cytoplasmic exon(s). Figs. 1D and 1E are drawn on the same scale.

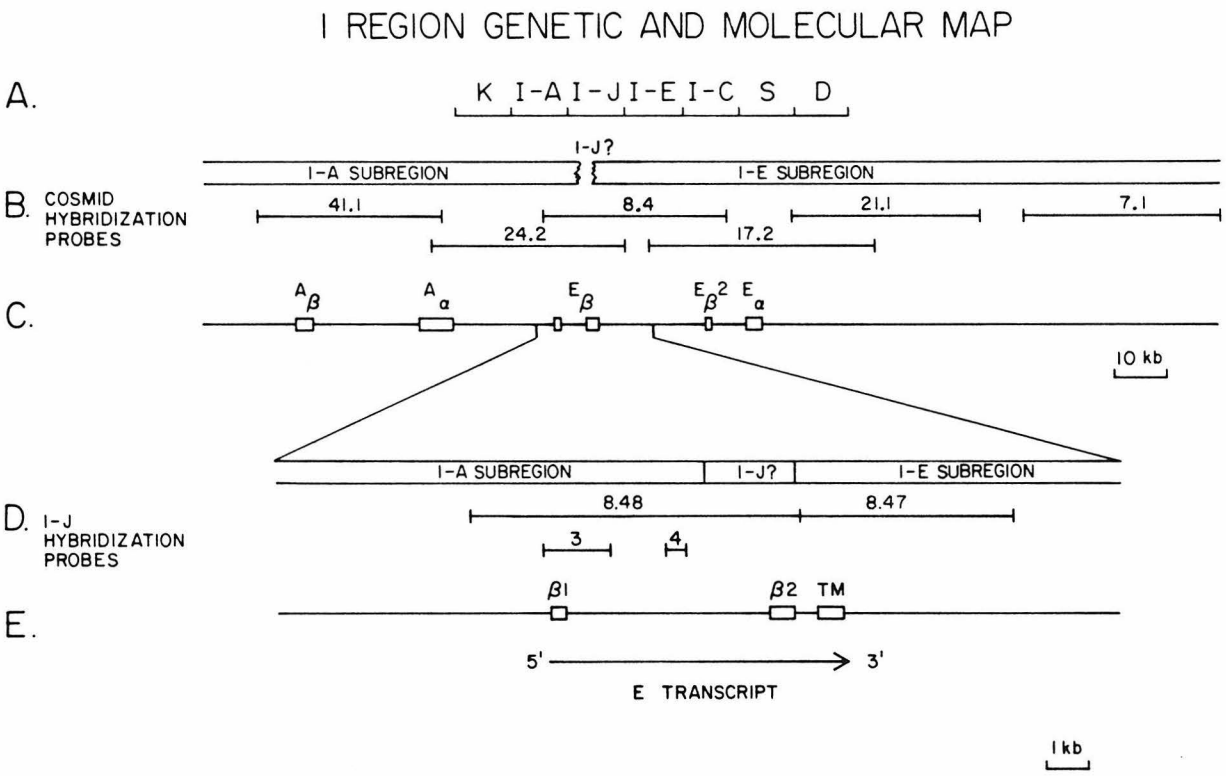
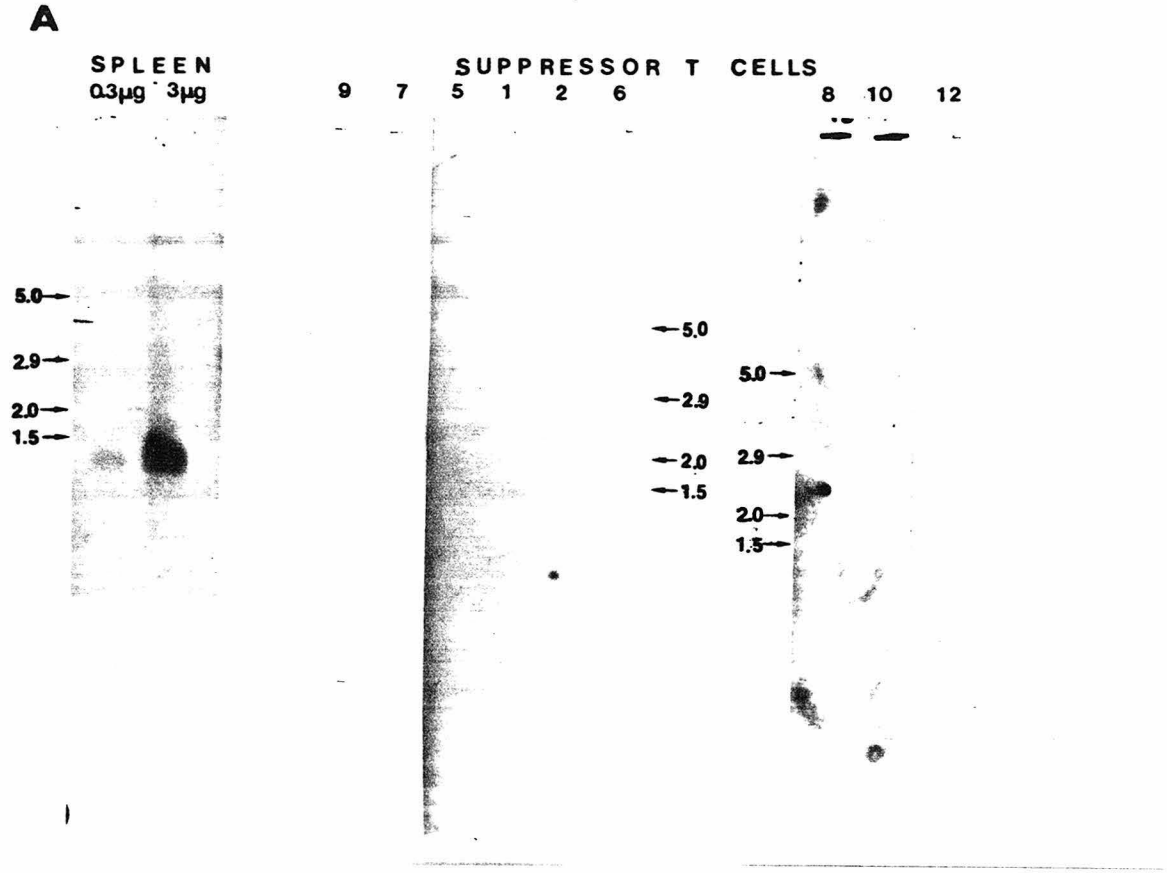

I REGION GENETIC AND MOLECULAR MAP
A.
K I-A I-J I-E I-C S D
I-J?
I-A SUBREGION
I-E SUBREGION
B. COSMID HYBRIDIZATION PROBES
41.1
8.4
21.1
7.1
24.2
17.2
C.
Aβ
Aα
Eβ
Eβ2
Eα
10 kb
I-A SUBREGION
I-J?
I-E SUBREGION
D. I-J HYBRIDIZATION PROBES
8.48
8.47
3
4
E.
β1
β2
TM
5'
3'
E TRANSCRIPT
1 kb

Figure 1

FIG. 2. (A) Hybridization of the 8.48 and 8.47 subclones to spleen poly(A)$^+$ RNA and to 10 μg of suppressor T-clel poly(A)$^+$ RNA. T-cell hybridomas are designated by the single or double digit numbers in the left-hand column of Table 2. RNA preparations on three separate filters were hybridized together with the I-J probes. (B) The same filter as in A, hybridized with pH-2IIa, a subclone from a cDNA encoding an H-2^d transplantation antigen. The migration distances of the molecular weight markers, mouse and Escherichia coli ribosomal RNA, and their lengths in kb are indicated.


A
SPLEEN
0.3µg 3µg
SUPPRESSOR T CELLS
9 7 5 1 2 6
8 10 12
5.0
2.9
2.0
1.5
5.0
2.9
2.0
1.5
5.0
2.9
2.0
1.5

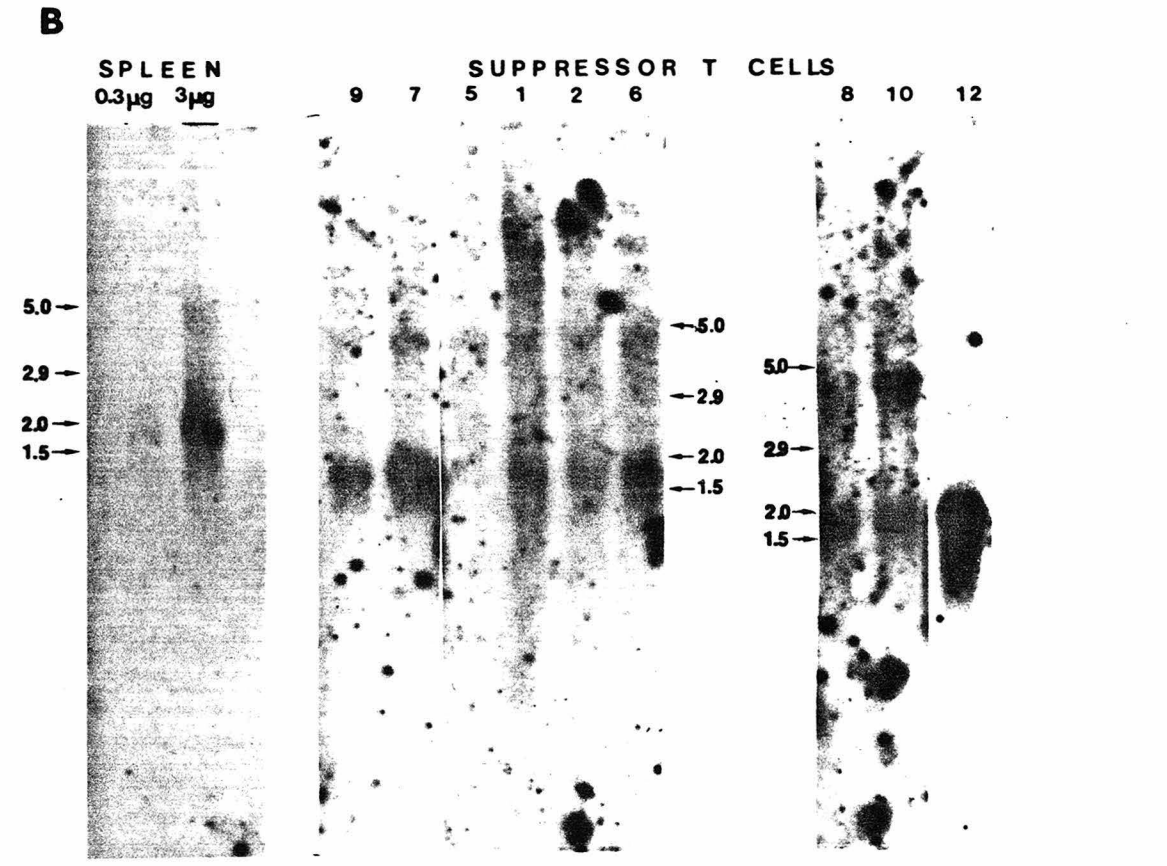

B
SPLEEN
0.3µg 3µg
SUPPRESSOR T CELLS
9 7 5 1 2 6
8 10 12
5.0
2.9
2.0
1.5
5.0
2.9
2.0
1.5
5.0
2.9
2.0
1.5

FIG. 3. Hybridization of cosmids 21.1 and 7.1 to poly(A)$^+$ RNA from spleen and T-cell hybridomas. Lane 1, 3 μg of poly(A)$^+$ RNA from BALB/c spleen. Lane 2, 0.3 μg of spleen poly(A)$^+$ RNA. Lane 3, suppressor 9 (372B3.5). Lane 4, suppressor 5 (368B1.5). Lane 5, AOD4 10.4 T helper hybridoma. Lane 6, DO11.10 T helper hybridoma. Lane 7, AODK 7.1 T helper hybridoma 10 μg of poly(A)$^+$ RNA from each of the T cells were tested.

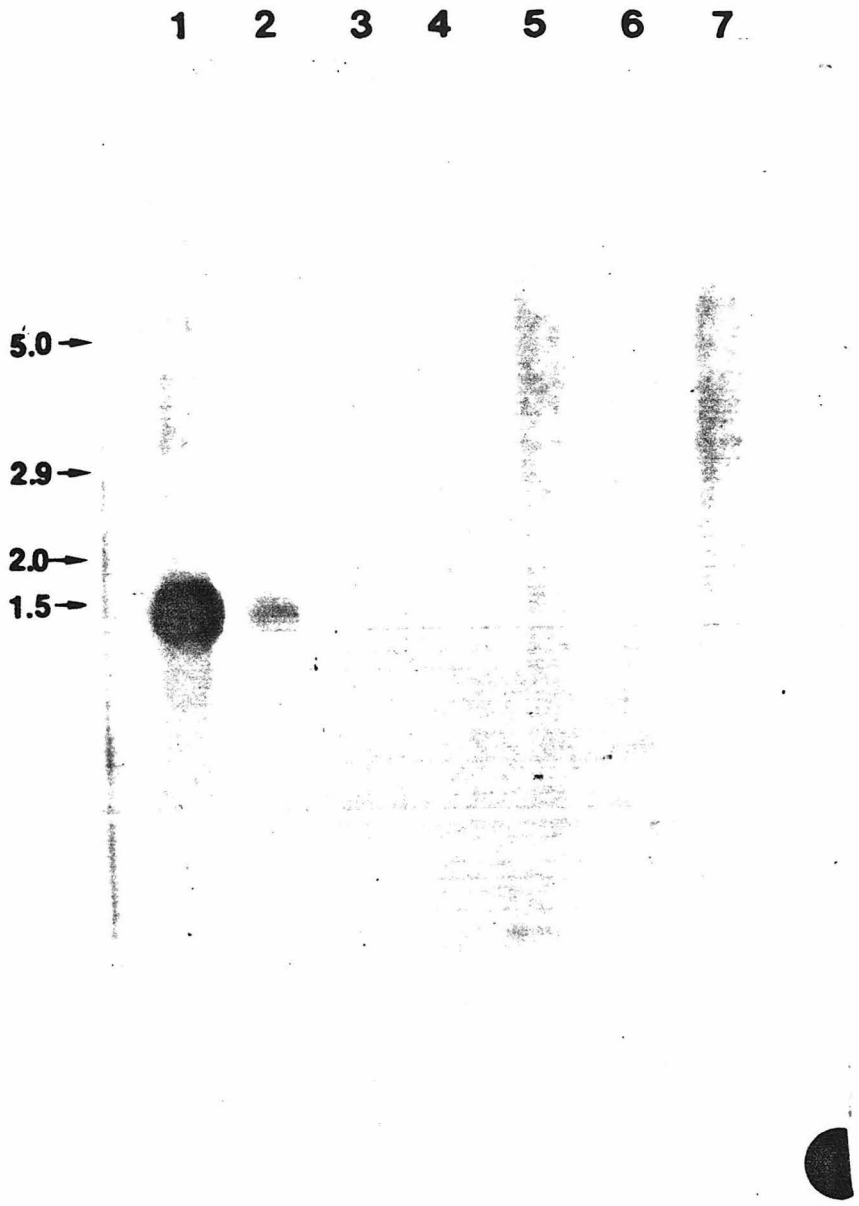
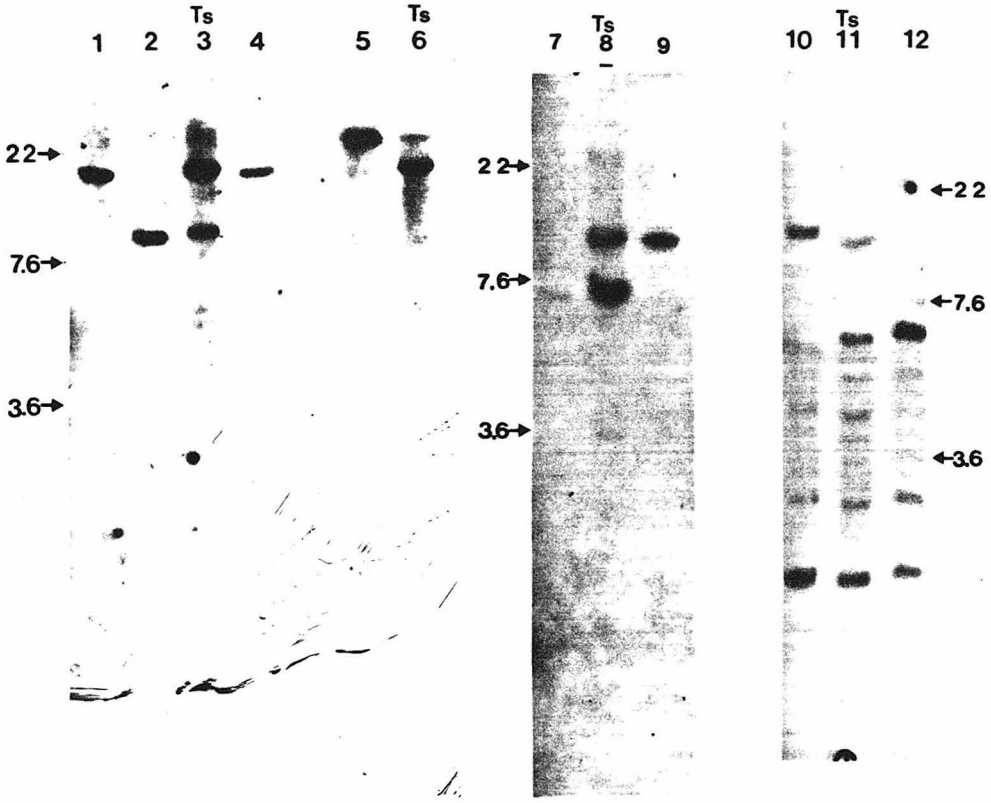

1
2
3
4
5
6
7
5.0
2.9
2.0
1.5

FIG. 4. Southern blots of T suppressor hybridoma DNA hybridized with I-J probes. Lanes 1-6 contain Kpn-digested DNA hybridized to probe 3. Lane 1 BW5147 thymoma ($H\text{-}2^k$), lane 2 B10 liver ($H\text{-}2^b$), lane 3 372B3.5 suppressor ($H\text{-}2^b/H\text{-}2^k$), lane 4 AKR liver ($H\text{-}2^k$), lane 5 B10.S liver ($H\text{-}2^s$), lane 6 395A4.4 suppressor ($H\text{-}2^s/H\text{-}2^k$). Lanes 7-9 contain BamHI-digested DNA hybridized to probe 3. Lane 7 AKR liver ($H\text{-}2^k$), lane 8 372B3.5 ($H\text{-}2^b/H\text{-}2^k$), lane 9 B10 liver ($h\text{-}2^b$). Lanes 10-12 contain EcoRI-digested DNA hybridized to probe 4. Lane 10 BW5147 thymoma ($H\text{-}2^k$), lane 11 366D3.2 hybridoma ($H\text{-}2^b/H\text{-}2^k$; this is an $I\text{-}J^b$ positive, GT-specific suppressor not described in Table 2). Lane 12 B10 liver ($H\text{-}2^b$). The migration distance of some molecular weight markers are indicated.


Ts
1 2 3 4
Ts
5 6
Ts
7 8 9
10 11 12
22
7.6
3.6
22
7.6
3.6
22
7.6
3.6
Kpn
Bam
RI

Chapter 7

CONCLUSION

WHY IS THE T CELL ANTIGEN-BINDING RECEPTOR ELUSIVE?

In retrospect, we might appear credulous having invested so much effort testing for expression of immunoglobulin genes by T lymphocytes. The hypothesis that T lymphocytes express immunoglobulin, primarily V_H gene segments, is based upon one of several possible interpretations of a body of experiments which all depend upon the detection of serologic cross-reactions between T cells and antibodies. We believe, however, that these data are cogent enough to have made the test of immunoglobulin expression the logical beginning for any analysis of the molecular biology of T-cell antigen-binding receptors. In fact, a similar approach has been taken in nearly a dozen laboratories around the world, and their results are largely consistent with ours.[1-9].

Advances in recombinant DNA technology have made it possible to isolate cloned sequences derived from genes which are expressed at low levels. For example, a cDNA clone can be obtained based upon partial amino acid sequence of the polypeptide encoded by that clone. The amino acid sequence can be reverse-translated into nucleotide sequence and a synthetic oligonucleotide primer complementary to a portion of the deduced nucleotide sequence can be used to identify the relevant clone. However, even with the most sophisticated instrumentation available, microgram quantities of purified material are required for amino acid sequencing.[10] A number of laboratories have attempted to obtain this amount of purified antigen-binding receptor from cloned T cells and with one exception (see below), have had no success.[11] It is also possible to obtain cDNA clones via assay of the protein product encoded by that clone.[12] Cloned cDNA(s) can be hybridized to RNA molecules and the specifically hybridized RNA can be translated <u>in vitro</u>. Some vector-host systems may permit both transcription and translation of the cloned segment, eliminating the need for hybridization selection of RNA. All of the above methods depend upon having a dependable assay for the T cell antigen-binding receptor. There are a number of potential assays which we consider below; unfortunately, none of these can be considered reliable.

1. Antigen binding. There are two potential problems with using antigen-binding to identify polypeptides that are involved in antigen recognition. First, although T cells clearly respond in a highly specific fashion to antigen, it has been difficult to demonstrate that they do in fact bind antigen. This difficulty could be caused by the presence of a small number of receptors on the surface of the cell, a low avidity of the receptor for antigen, or finally because the antigenic determinant itself is not well defined. In a number of experiments, T cells apparently could bind antigen on the surface of macrophages or other cells, but could not bind free antigen.[13,14] The binding to cell-surface antigen is restricted to cells with the proper MHC haplotype, which suggested to some workers that only a complex of antigen and MHC (altered-self), as opposed to free antigen, is actually recognized by the T-cell receptor.[13,14] There are, however, a few exceptions in which T cells apparently do bind antigen,[15-18] and soluble factors derived from T lymphocytes often will bind to antigen affinity columns.[19] Currently, the controversies concerning the existence of T lymphocytes capable of binding free as opposed to macrophage-adsorbed antigen remain unresolved. The second problem concerns the possibility that some other cellular protein(s) will bind antigen with relatively high affinity. This is particularly serious for the "academic" antigens preferred by immunologists which are often small, highly charged or aromatic haptens. A polypeptide binding azobenzene arsonate (Ars) synthesized by a presumed Ars-specific T cell hybrid, is not an antigen-binding receptor as originally reported,[20] and may in fact be an enzyme involved in phosphate metabolism.[21] Polypeptides synthesized by a GAT-specific T cell hybridoma and a control T lymphoma which bind a GAT Sepharose affinity column have been analyzed by gel electrophoresis. In this case, both the antigen-specific cell and the control T lymphoma synthesized a large number of proteins which bound to the affinity column and the gel patterns are quite similar for the two samples tested.[22] Another group has reported that some GAT specific T cell hybridomas bind

significantly more radiolabeled GAT than the control cells do. Unfortunately, some KLH-specific T cell hybridomas and T lymphomas also bind increased amounts of GAT.[23]

2. Antisera. A number of laboratories have attempted to generate monoclonal antibodies against the T-cell antigen-binding receptor. Following immunization with cloned T cells, antibodies produced by hybrid B cells are assayed for their ability to alter T-cell function. However, this is a poor criterion for defining anti-receptor antibody, since the functional effect could be caused by steric hindrance if the antibody recognizes a molecule found in close proximity to the receptor. It is also possible that the antibody could act by altering the mobility and/or distribution of cell-surface components not specific for antigen, but important for the activation of lymphocytes. In fact, most of the monoclonal antibodies which interfere with T-cell function do not recognize the antigen-receptor. At least four separate laboratories screened for monoclonal antibodies which interfered with cytolytic T-cell function, and in 17/20 cases the antibodies recognized the murine Lyt-2,3 molecular complex (or its human equivalent).[24-27] However, this protein appears to be a differentiation antigen rather than an antigen receptor since the Lyt-2,3 molecules display little charge heterogeneity when analyzed on two-dimensional gels.[28] In addition, there are some antigen-specific cytotoxic T cell clones which do not express the Lyt-2 specificity.[27] Two other monoclonal antibodies which inhibited cytotoxic T-cell function recognize a molecule (LFA-1) which is not likely to be the T-cell antigen-receptor since it is present on B cells and bone marrow cells as well as T lymphocytes.[25,27,29] The final monoclonal antibody has not yet been characterized. At least two groups have generated antibodies specific for particular cloned T cells.[30] These "anti-idiotypic" antibodies can even distinguish between cloned helper T cells responding to the same antigen, and it is possible they recognize the variable portion of the T-cell antigen-binding receptor.[30] Very recently, monoclonal antibodies

specific for particular T cell clones have also been obtained.[31] As noted above, assays which measure the effect on a complex cellular response are difficult to interpret, and it is also possible that different cloned cells may display the same molecule differently on the cell surface. For example, only 59/115 mouse cytolytic T cell lines could be inhibited by monoclonal antibody directed against Lyt-2,3, despite the fact that all the cells tested expressed roughly equivalent amounts of the Lyt-2,3 molecule.[32] Blocking of cytotoxicity by the antibody did not correlate with either the antigenic specificity or the lytic activity of the T cell lines. Similar results have been reported with human T cell lines.[26] It is possible, then, that some of the clone-specific antibodies will recognize not the T-cell antigen-receptor, but molecules expressed on very small subsets of T lymphocytes which are otherwise quite analogous to Lyt-2.

In order to avoid generating a large number of B-cell hybridomas making antibodies against T lymphocyte antigens which seem to be immunodominant such as Thy-1 or Lyt-2, a number of investigators have used congenic strains which have limited differences in their genetic background for their initial immunizations. The most conspicuous success has been achieved using mice congenic for the heavy chain constant region locus.[33-35] Three laboratories have obtained monoclonal antibodies that recognize a set of T cell antigens linked to the constant region (Ig-1) locus.[35-37] Monoclonal antibodies specific predominantly for thymocytes (Tthy)[38], helper T cells (Tind)[35-38], suppressor T cells (Tsu)[35-38] and recently for killer T cells[35] have been defined. The Tsu determinant has been mapped between the C_H and prealbumin genes.[39] The gene order is V_H-C_H-Tsu-Pre. The antibodies can perturb T-cell functions, immunoprecipitate proteins and react with antigen-specific soluble factors. It has been hypothesized that these alloantigens are the constant region of the T-cell antigen-binding receptor. The data indicating that these antibodies can recognize determinants on the antigen-receptor, however, are very limited. The monoclonal

antibodies bind to molecules in crude extracts of T cells that contain either augmenting (helper) or suppressing T cell factors.[35,37,40,41] However, the physiological significance of these factors is not clear, since they have a very small effect on the hapten-specific B cell response. In addition, a recent study showed that in a series of T-cell hybridomas, cell-surface expression of Tind and Tsu is not correlated with expression of Lyt antigens.[38] Since Lyt antigen expression is correlated with T-cell function, this raises questions concerning the functional significance of the Ig-1 linked T cell alloantigens.

3. Biological Assay. Direct functional assay of genes encoding antigen-binding receptors must await the isolation of clones encoding the T cell antigen-receptor or techniques for the efficient transfection of lymphocytes. However some cloned T cells synthesize protein molecules (factors) which bind specifically to antigen affinity columns and can replace the antigen-specific function of the T cell in vitro.[19] Thus, hybridization-selection schemes can be employed to clone the gene(s) which encode the factor(s). The factors, however, are very poorly characterized. Some reported molecular weights are 80,000, 65,000, 34,000 and 24,000 daltons for the antigen-binding chain.[17-19,42-44] A second chain which may contain I region encoded determinants is sometimes present.[17,42-45] In one instance, the antigen-binding site, idiotype and I-J determinants are reported to be present on a single 24,000 dalton polypeptide.[43] We must question whether the diversity in the molecular properties of the factors reflect genuine T cell heterogeneity or experimental artifact. Moreover, the biological significance of the factors is not without question. Although it is difficult to compare systems for the generation of in vitro immune responses to different antigens, the reported biological activities of suppressor factors can vary over three orders of magnitude.[46-47] While many of the soluble factors are antigen-specific, it is conceivable that some are immunologically-irrelevant proteins that bind to or hydrolyze the antigen in question, thereby efficiently removing it from culture

and depressing the immune response to that particular antigen. In this regard, it is noteworthy that the partial amino acid sequence of the best characterized suppressor factor is virtually identical to the abundant high density lipoprotein Apo-A1.[48]

In summary, at the functional level the T-cell antigen receptor is reasonably well understood (Chapter 2), while at the structural level it remains a mystery. Currently, there is no assay (aside from measurement of the antigen-specific responses of T lymphocytes) which can unambiguously identify this molecule. In the above discussion, we have considered some of the difficulties involved in defining the T-cell antigen-binding polypeptide. It is possible that a combination of techniques, including serological assays using monoclonal antibody and functional studies will permit the problem to be solved. As we learn more about the structure and function of MHC gene products and the fine specificity of H-2 restricted T cell responses, the questions concerning the T-cell antigen receptor become even more tantalizing. Hopefully the elusive receptor will soon be captured.

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