MECHANISMS OF LYMPHOCYTE ACTIVATION AND IMMUNE REGULATION

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PREFACE

During the past 5 years rapid progress has been made in the understanding of biochemical pathways for signal transduction in lymphocyte activation. Gene cloning technology has been instrumental in defining and making available in pure form of a number of growth and differentiation factors, in the characterization of their receptors, and in the delineation of genes for the T cell receptor. This book is divided into 6 sections. Section 1 deals with the molecular structure of the T cell receptor. Section 2 discusses the role of the T cell receptor, membrane ion channels and biochemical pathways of signal transduction in T cell activation. The molecular structures and biological and immunological effects of interleukin 1, interleukin 2 and interleukin 3 are presented in Section 3. This section also details the structure of interleukin 2 receptor and its use as a target for therapy for certain leukemias. Section 4 includes the biochemical events which occur following the delivery of the signal for B cell activation, proliferation, and differentiation by antigen, growth/differentiation factors. The molecular structure of B cell stimulating factors is also discussed. The role of oncogene expression in cellular activation and differentiation is included in Section 5. The cellular and molecular basis of natural killing and the molecular basis of cyclosporin A-mediated immunosuppression are discussed in detail in Section 6.

We hope this book will serve as a reference work on basic mechanisms of lymphocyte activation, proliferation, and differentiation for immunologists and molecular biologists.

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THE T CELL RECEPTOR: ITS REPERTOIRE AND ROLE IN THYMOCYTE DEVELOPMENT

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Although the discovery of the genes and protein of the T cell receptor has solved some immunological problems many issues remain unsolved. These include questions of tolerance, the relationship between the structure and specificity of the T cell receptor and an understanding of the selective processes which occur in the thymus.

Two of these issues will be addressed in this paper, the role of the receptor in thymocyte differentiation and a question raised by Jerne some years ago, about the germ line T cell repertoire (1). Our data suggest that, not unexpectedly, the receptor is indeed involved in thymocyte maturation, at or just after the point at which immature thymocytes are selected to become mature. This indicates that most of the proliferation which occurs in the thymus in immature cells is not receptor-mediated, even though many of these cells bare receptors. Moreover it suggests a stage in the life history of thymocyte upon which immunologists interested in self MHC-restriction and tolerance induction should focus.

In the second part of this paper we show data which indicate that Jerne was correct in hypothesising that the germ line repertoire of T cell receptors is indeed directed against MHC antigens.

MATERIALS AND METHODS

Animals

BALB/cBy and C57B/6J mice were purchased from the Jackson Laboratory. Timed pregnant females were obtained from the Jackson Laboratory or raised in our own vivarium. The day of finding a plug was designated day 0 of pregnancy.

Production of T cell hybridomas and cell cultures

Antigen-specific, MHC-restricted T cell hybridomas were produced, cultured and characterized as previously described (2,3). Variants were produced by cloning at limiting dilution after prolonged culture, or after 500-750 rads or $^{\gamma}$-irradiation (4).

Production and use of anti-receptor antibodies

The anti-receptor monoclonal antibodies KJ-1, KJ-12, and KJ-16 have been described previously. KJ-1 binds to an idiotypic determinant on the T cell hybridoma DO-11.10, specific for chicken ovalbumin (OVA)/IA^d, OVA/IA^d and IA^b alone (5). KJ-12 binds to an idiotypic determinant on the T cell hybridoma 3DT-52.5, specific for D^{d6}. The rat monoclonal antibody KJ-16 binds to a determinant on a family of VB proteins, variously called C5, Vβ4 or Vβ8, expressed by 15-20% of T cells in BALB/c and C57Bl/6 (7-13).

KJ-16 ascites was raised in BALB/c mice. F(ab')2 fragments were prepared by pepsin digest, after which they contained about 13% intact immunoglobulin molecules. Following reduction and alkylation and gel filtration and F(ab')2 or IgG.

Fetal thymus organ culture

Thymus lobes from d14-d17 fetal mice were cultured as organs on rafts as previously described (14) for varying lengths of time.

Cytofluorografic analysis of thymocytes

Cells were incubated with anti-Thy 1, T24/40 (a gift from Dr. I. Trowbridge); anti-L3T4, GK1.5 (a gift from Dr. F. Fitch); anti-Lyt-2, 2.43.1 (gift from Dr. F. Fitch) or KJ-16 followed by staining with fluoresceinated anti-rat κ RG-7 (a gift from Dr. L. Arnold). Analysis was carried out on an Ortho Cytofluorograf as previously described (15). Controls included unstained cells, and cells incubated with RG-7 alone. Dead cells were gated out with propidium iodide.

Production, cloning and sequencing of cDNA

cDNA libraries were prepared in pUC9 from DO-11.10 and 3DT-52.5 as previously described (16). T cell receptor - encoding clones were picked by hybridisation previously described C α [4] and Cβ [17] probes. After subcloning in pEMBL9 DNA was sequenced by the dideoxy method (18,19).

RESULTS AND DISCUSSION

The role of the receptor in thymocyte differentiation

At least 2 processes involving the receptor or receptor-like molecules are known to occur in the thymus, during the development of T cells. These are selection for self-MHC restriction, such that T cells which emerge from the organ are selected to react with antigen in association with MHC products expressed within the thymuses, and tolerance induction (20-22). Both of these are crucial and mysterious processes in the immune response, the solution of which will be important both theoretically and in practice.

Expression of receptors on thymocytes

In an attempt to establish the facts upon which any solution of these problems must be founded, we have studied the expression of receptors on thymocytes, both by cytofluorografic analysis, and at the ultrastructual level. Receptors in these experiments were identified using the anti-Vβ family antibody, KJ-16. This antibody recognizes and precipitates intact receptor α / β dimers from the surfaces of thymocytes and peripheral T cells (15), and also binds to and precipitates free intracellular chains from certain cell types including immature thymocytes (Dr. C. Hannum, unpublished observations). The antibody binds to about 20% of peripheral T cells. There is not evidence that there is a preference for the expression of a particular VB ontologically or differentiationally. It is therefore probably valid to assume that our data with KJ-16 are representative of all receptor expression, and thus numbers and percentages obtained with this antibody can be multiplied by 5 to approximate data for all receptors.

Staining of adult thymocytes with KJ-16 gives a trimodal curve (Figure 1A). Most of the cells are unstained, because they bare receptors which do not react with KJ-16, or because they bare no receptors at all. About 10% of the cells stain at variable and low levels and about 2% of the cells stain with a density similar to that of peripheral T cells. A related profile is found when human thymocytes are stained with anti-T3 (Figure 1B). In this case a large percentage of the cells (about 60%) stain at variable and low levels, and a much smaller percentage (about 14%) at mature T cell levels.

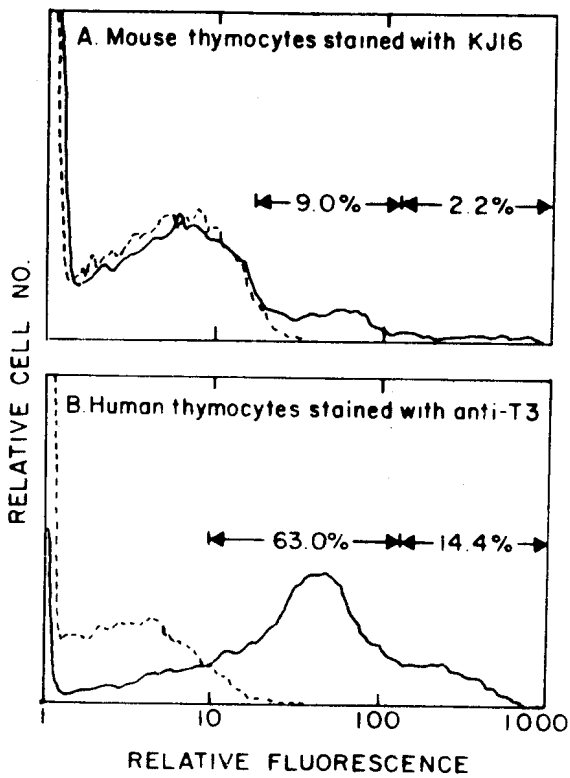

Figure 1. Staining of mouse and human thymocytes with anti-receptor and anti-T3 reagents.

A. Adult BALB/c thymocytes were incubated with KJ16 followed by FL-RG7 (——). Controls were unstained (- - -) or incubated with FL-RG alone (not shown, but undistinguishable from the unstained control). Numbers and bars indicate the percentages of cells stained with low or high numbers of receptors/cell. Cells bearing high numbers of receptors were defined as within 2 standard deviations of the mean of receptor numbers on a peripheral T cell population stained on the same day.

Table I

Presence of Receptor on the Surface of Different Thymocyte Populations in Adult Mice

Population	% Surface Staining with KJ-16	Approx. % surface receptor position	Level of receptor	Rate of appearance of receptor-bearing cells in ontogeny	Remarks
Immature (cortical) thymocytes	10-14%	50-70%	low, variable	fast (d17-20 of mouse fetal life)	Cells may also contain internal chains. A few cells have receptors capped on epithelial
Mature (medullary) thymocytes	20%	100%	peripheral T cell levels	slow (approx. 0% at birth reaching adult levels after 4 weeks)	no receptor capping seen
Peripheral T cells	20%	100%	peripheral T cell levels	slow	-

B. Fetal human thymocytes were stained with FL-anti-T3 (——) or were unstained (- -). Numbers and bars indicate the percentage of cells staining with low or high numbers of receptor/cells defined as described above.

We have previously shown that mature, cortisone resistant, peanut agglutinin-(medullary) thymocytes bind KJ-16 with the same frequency, and in the same amounts as peripheral T cells (15). These are presumably the high density staining cells in the whole thymocyte profiles described above. This result suggests that the frequency of mature thymocytes which have successfully rearranged and expressed both α and β chains is similar to that of peripheral T cells. Moreover, at the ultrastructural le el these cells bare receptor distributed evenly over their surfaces, and these molecules are not capped at any point on the cells, or in association with any other cell type (23).

By contrast, the percentage of immature, peanut agglutinin positive (cortical) cells which react with KJ-16 is 50-70% of that of peripheral T cells (10-14% in absolute terms), and these cells bare on their surfaces very variable and low levels of the receptor, averaging 1/5th the numbers of peripheral T cells and mature thymocytes (15). These are the low density staining cells shown in Figure 1. This suggests that about half of the cells in the immature thymocyte pool have successfully rearranged and expressed both $\alpha$and βchains. This may seem like a very high percentage given that, because of out-of-frame rearrangements, only about one in three $\alpha$and βchain genes should be functional. The cell has several opportunities to construct functional genes, however, both because of multiple V and J (atα) and V,D and J (at β) loci for each collection of genes.

In any case, our data show that a substantial proportion of immature cells have successfully accomplished the task of expressing receptors. Given the high frequency of immature cell death, this implies that although some cortical thymocytes may die because they have failed to express receptors, other selective processes must also be involved in causing cell death, perhaps including those described above.

At the ultrastructural level it has been very difficult to observe surface receptor positive cells in the thymus cortex, probably because they bare so little receptor that not enough will be present in a given thin section of a cortical thymocyte to be apparent. Two types of cortical, KJ-16$^+$ cells have been seen with the electron microscope, however. The first, and most common, has internal reactive material, concentrated in the perinuclear region. Our preliminary experiments indicate that this may be composed of free β chains. The second, rarer, type of cell does bare surface receptors, and these are detectable because they are capped in that region of the thymocyte which is in contact with a cortical epithelial cell (23).

The identification of low receptor density cells as immature, and high receptor density cells as mature thymocytes was confirmed by an ontological study. This showed that receptor first appears in the mouse thymus on d17 of fetal life. Receptor-bearing cells rapidly increase in number thereafter, reaching about 50% (10% KJ-16$^+$) by d20 when the mouse is born. These cells all bare low levels or receptor not reach adult, steady state numbers, (20% of all thymocytes, 4% KJ-16$^+$) until after the mouse is 4 weeks old. These data are summarized in Table 1.

The conclusions from these observations are as follows. Prothymocytes enter the organ and rearrange and express their receptor and receptor-like genes (15,17,24-26). Receptor first appears on immature, cortical thymocytes at low levels, and these cells appear rapidly in the developing mouse (15). A few of these cells cap their receptors on epithelial cells, perhaps because of interaction of their receptors with MHC molecules on these target cells. Electron micrographs demonstrating this may be, in fact, visualizations of the process of selection for self MHC-restriction (23). Mature, medullary thymocytes appear slowly in the developing animal, produced, perhaps, as a consequence of immature thymocyte/epithelial cell interaction. These mature cells bare high, peripheral levels of receptor and of course are similar to peripheral T cells both in the surface markers they bare, and in the fact that they function in immune responses.

The receptor is needed for the appearance of mature but not immature thymocytes

Since the receptor is present at two different stages of the thymocyte's life history, selection for self MHC restriction and/or tolerance may occur at either of these stages. In order to find out which processes in thymocyte maturation require the receptor, experiments were carried out in which the effects of a blocking, anti-receptor antibody (KJ-16) on thymocyte development were observed. In these experiments immature and mature thymocytes were defined by their receptor densities as described above.

We approached these experiments in 2 different ways, the inhibitory antibody was either added to fetal thymus organ cultures (d17 - onwards or mice were injected daily or twice daily after birth with large doses of F(ab')2 or F(ab') preparations of the antibody respectively. The results of these two different kinds of experiments were in fact similar and led to identical conclusions.

We and others have shown that thymocyte development precedes fairly normally in fetal thymus organ cultures, but not in suspension cultures for a short lag, and mature, high receptor density cells appear. The main difference between processes in vivo and in vitro seems to be due to the lack of a continuous supply of precursor cells and an accumulation of mature cells in the latter system. Over the course of a week or two's culture of, for example, d17 thymuses, immature cells gradually disappear and $L3T4^+$ or $Lyt\text{-}2^+$ "single positive" cells with high receptor densities appear. This is an understandable problem for an organ which is now isolated from the whole animal and therefore from a source of stem cells (28).

Culture of d17 fetal thymus lobes for 4-8 days in the presence of KJ-16 has little effect on the cell yield. KJ-16-reactive immature and mature cells are, however, completely absent from such cultures. If the KJ-16 antibody is washed out, however, and the thymocytes cultured for a further 24 hours, KJ-16-reactive immature thymocytes reappear. KJ-16-reactive mature thymocytes do not (Table II).

Table II

Anti-receptor Antibody has no effect on Immature cells

but prevents the appearance of mature thymocytes

Conditions	Antibody administered	% $KJ16^+$ Immature thymocytes	% $KJ16^+$ Mature thymocytes
d17 thymus lobes cultured 7 days	none	10.3	1.3
d17 thymus lobes cultured 7 days	KJ-16	12.2	0.3
25 day old BALB/c	none	-10	2.1
25 day d BALB/c	KF-16 $F(ab's)_2$ daily since birth	-10	0.2

A similar but not identical phenomenon is observed if KJ-16 F(ab')2 or F(ab') is administered continuously to baby mice (29). This procedure coated $KJ\text{-}16^+$ cells with antibody, rather than capping their receptors from their surfaces, since cells from such

animals stained with the secondary reagent FL-RG7 without prior incubation with KJ-16. Cells were harvested from the thymuses of these animals in normal numbers and with normal distributions of Thy-1, L3T4 and Lyt-2. Although cells bearing low densities of KJ-16-reactive receptors were unaffected in their numbers by the antibody, in a result reminiscent of the in vitro experiments, mature cells bearing high densities of receptors were absent (Table II). This was even more dramatically apparent in animals treated 2 days before sacrifice with hydrocortisone. Such animals contain only mature cells in their thymuses, and, in antibody treated mice, KJ-16-reactive thymocytes were completely absent (data not shown). Similar results were obtained from mice treated with F(ab'). Incubation of thymocytes for 2 days in suspension culture did not allow the reappearance of KJ-16$^+$ mature cells, showing that receptor was not simply capped from their surfaces. Controls indicated that these results were not due to artifactual and mature cell-selective cytotoxic processes such as opsinisation or complement-mediated killing.

The inescapable conclusions from these experiments include the surprising idea that immature thymocytes, even though they may bare surface receptor, are not dependent on this molecule for their existence, maintenance or expansion. They are unaffected in numbers by antibody-mediated capping or blockade. The antibody does not stimulate selective expansion of these cells. This suggests that at this stage normal receptor-mediated signalling mechanisms are not operating. Since these cells are also nonresponsive to Concanavalin A and/or interleukin 2, this is not, perhaps, so unexpected.

By contrast the existence of mature thymocytes seems to depend on a receptor mediated-process either in their production, or at some stage relatively soon after their appearance.

Taking all our experiments together we would like to suggest that mature thymocytes are selected from the large immature pool on the basis of the fact that the receptors on a small proportion of immature thymocytes can react with (MHC) molecules on thymic cortical epithelial cells. KJ-16, by interfering with this process, interferes with the production of mature, but not immature KJ-16-reactive thymocytes.

T cell repertoire

A number of questions related to the T cell repertoire remain unanswered. These include the relationship between receptor sequence and specificity for antigen and MHC, a problem addressed by another speaker at this meeting (see Hedrick et al.) and the suggestion made some years ago by Jerne (1), that the germ line repertoire of the lymphocytes of any given species would be designed to recognize the MHC antigens of that species. In the second part of this paper we would like to report some results related to these two points.

Relationship between T cell receptor structure and specificity.

It is now widely recognized that the repertoire of receptor Vβs in mice and man is quite limited. So far only about 20 Vβs have been described in mice, and the total number is small enough that some are found with high frequency, and the family of 3 Vβs recognized by KJ-16 is used by 20% of all T cells in most mouse strains. It is therefore not surprising that the same Vβ protein is used by T cells with receptors of widely different specificities, and can be involved in recognition of structures as different as Class I and Class II molecules (15).

The repertoire of chains seem to be a lot larger. There are probably more than 50 Vαs and 50 Jαs. Hopes for discrimination between receptors which are Class I or Class II-restricted therefore rested on this chain. We have investigated the structure of receptors specific for the Class II molecule, IA^b and the Class I molecule, D^d on the T cell hybridomas DO-11.10 and 3DT-52.5 respectively. This is not such a straightforward task because, unlike normal T cells, T cell hybridomas made by fusing T cells to the AKR thymoma, BW-5147, contain functional receptor polypeptides derived not only from the normal parent, but also from the tumor cells. Any given hybridomas may therefore bare up

Table III

Properties of 3DT-52.5 and 2 of Its Subclones

Hybridoma	mRNA for:								Response to:		
	V	3DT	V	3DT	V	BW	V	BW	Dd	polyvalent KJ-12 (anti-idiotype)	polyvalent KJ-16 (anti-allotype)
3DT-52.5	+		+		+		+		+	+	+
3DT-52.5x13	+		+		-		+		-	-	+
3DT-52.5x25	+		-		+		+		-	-	-

to 4 different receptors due to the various combinations of these α and β chains. Our previous data have suggested that the DO-11.10 receptor for IA^b is made up of $KJ\text{-}16^+$ β chain derived from the normal T cell parent of the cell, and an α chain derived from BW-5147.

Similar studies were performed on 3DT-52.5. Two anti-receptor antibodies react with this hybridoma, KJ-12, which recognizes an idiotypic determinant on one of the receptors of the cell, and KJ-16. KJ-12 inhibits recognition of D^d by this hybridoma, suggesting that KH-12 binds to the receptor for D^d. KJ-16 cannot be used in this type of analysis for this cell because in soluble form it stimulates the hybridoma to secrete IL-2. In polyvalent form both KJ-12 and KJ-16 stimulate IL-2 production by the cell line.

3DT-52.5 contains functional mRNA from α and β chains derived from both the normal T cell and BW-5147. In order to find out which chains were involved in the D^d receptor 3DT.52.5 was x-irradiated with 500 and 750 rads. For each radiation dose only a few cells survived. These were grown up, cloned and characterized. A summary of the properties of 2 subclones is shown in Table III.

3DT-52.5.x25 does not respond to D^d, nor to polyvalent challenge by either of the 3DT-52.5-binding anti-receptor antibodies, KJ-12 and KJ-16. The hybridoma contains mRNA from all parental α and β chains except the normal T cell parent-derived β chain, distinguished by a probe for Vβ3DT. Loss of the gene coding for this mRNA was confirmed by Southern blot. 3DT-52.5.x13 has therefore lost the ability to recognize D^d because of loss of the normal T cell-parent derived β chain.

3DT-52.5x13 also no longer responds to D^d and polyvalent KJ-12, but does respond to KJ-16, directed at the β chain involved in the D^d receptor. It appears that this subclone has lost the α chain of the D^d receptor. Northern and Southern blots proved that the α chain lost was derived from BW-5147, and not from the normal T cell parent. These subclones allow us to conclude that the receptor for D^d on 3DT-52.5 is made up of the α chain encoded by BW-5147 and a β chain derived from the normal T cell parent.

The sequence of the BW-5147 α chain has already been published. We cloned and sequenced the β chains of DO-11.10 and 3DT-52.5. DO-11.10 β is made up of Vβ8.2, Dβ1.1, Jβ1.1; 3DT-52.5 β is made up Vβ8.1; Dβ2.1; Jβ2.3.

The two receptors for IA^b and D^d respectively share exactly their α chains. The β chains differ at 16 positions scattered throughout the chains but concentrated in predicted hypervariable regions, particularly at the VDJ junction. From these observations we conclude that, like Vβ. the α chain does not determine even a feature of the receptor as fundamental as its restriction for Class of MHC. Also neither α and β chain impose absolutely on the specificities of the receptors they compromise, and even molecules as different as Class I and Class II products can be bound by relatively similar receptors. An extension of these conclusions is that Class I and Class I molecules may have quite similar gross structures, as predicted by others.

The T cell germ line receptor repertoire recognizes MHC

As mentioned above, it has been suggested that germ line (T cell) receptors recognize MHC proteins of the species. This suggestion accounts very nicely for the fact that a high percentage of peripheral T cells respond to allogeneic MHC and, of course, self MHC plus antigen. Alternatively, one could account for the peripheral T cell data by invoking the powerful selection mechanisms known to act in the thymus. The germ line T cell repertoire might have random specificities, but selection for self-MHC restriction in the thymus might simultaneously select for recognition of allogeneic MHC at high frequency in the periphery.

In T cell hybridomas, receptors form by random combination of tumor cell and normal cell polypeptides which have not been selected together in the thymus. Although each chain may have been selected as part of the original receptors of the parental cell, the "mixed" receptors formed by complementation in the hybrid have not. In fact, for

Table IV

Examples of T Cell Hybridomas with Allogeneic Reactivity
Due to Receptor polypeptide Complementation

T cell hybridoma	Specificities: Ag + self	Specificities: MHC	(Presumed) Composition of anti-MHC receptor	
DO-11.10	OVA/IA^d OVA/IA^b	IA^b	BW	DO
3DT-52.5	-	D^d	BW	3DT
3DO-54.8	OVA/IA^d	$IA^s IA^f$	DO	BW
AODH-7.1	HgG/IE^d	IA/E^d	?	AODH
8DO-131	OVA/IA^d	Mls	8DO	BW