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The detection of F(ab′)$_2$-related surface antigens on the thymocytes of children

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Summary. A fowl antiserum to human polyclonal F(ab′)$_2$ with specificities for variable region antigens of both light and heavy chains and for the constant portion of the Fd fragment bound to about 20 per cent of thymocytes from three children. Binding was completely inhibited by purified human polyclonal IgG and only partly by L chains. The fowl anti-F(ab′)$_2$ also bound to a subpopulation of peripheral blood lymphocytes which was somewhat larger than the B-cell population identified with other anti-immunoglobulin sera. Binding was detected by autoradiography and the amount of radiolabel seen on thymocytes was nearly as great as that on peripheral blood lymphocytes.

As no binding to thymocytes was seen with other anti-immunoglobulin sera we propose that the fowl anti-F(ab′)$_2$ recognizes immunoglobulin or material with structural similarities which is synthesized by thymocytes but of which predominantly V region determinants are exposed on the membrane surface.

INTRODUCTION

The nature of the receptor for antigen recognition by T lymphocytes is still debated although it is known that immunoglobulin (Ig) is the receptor on B cells (Warner, 1974). Ig has been detected under certain conditions on the T-cell surface but in minute amounts compared with B cells (Nossal, Warner, Lewis and Sprent, 1972), and there is controversy about whether this Ig is endogenously synthesized by the T cells themselves (Hudson, Sprent, Miller and Playfair, 1974; Hunt and Williams, 1974; Haustein, Marchalonis and Crumpton, 1974).

The best evidence to date for Ig as the T-cell receptor for antigen comes from the work of Roelants *et al.* (Roelants, Forni and Pernis, 1973; Roelants, Ryden, Hagg and Loor, 1974). With polyvalent anti-Ig sera they were able to induce capping of the T-cell receptors for highly radiolabelled antigens. After the T cells had been cultured *in vitro* the receptors regenerated indicating that they were endogenously synthesized. Final proof that the receptors were Ig was lacking, however, because in the hands of these authors immunofluorescence was too insensitive to directly visualize Ig on the T cells.

Certain conditions appear to be essential to demonstrate Ig on T cells or thymocytes. A sensitive technique is important, such as autoradiography (Nossal *et al.*, 1972), highly sensitive immunofluorescence (Santana, Wedderburn and Turk, 1974), inhibition of antigen binding (Hammerling and McDevitt, 1974) or cytotoxicity (Burckhardt, Guggisberg and von Fellenberg, 1974), and even with optimum use of these methods some but not all anti-Ig sera are effective. Therefore the specificity of the anti-Ig antiserum is of prime importance. Antisera raised to polyclonal Ig are often effective, whereas those to monoclonal proteins frequently are

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not (Burckhardt *et al.*, 1974). We have used an antiserum raised in fowls against polyclonal human $F(ab')_2$ prepared from IgG which after suitable absorptions reacts with the Fd of human IgG, with V regions of light chains (Orlans and Pearce, 1974) and with a high proportion of monoclonal μ and α heavy chains. It also reacts with Ig of other species such as mouse and rabbit indicating a broad spectrum of specificities for mammalian Ig determinants. We have found that this antiserum will bind to about 20 per cent of human thymocytes, but our conditions do not reveal binding to circulating T cells.

MATERIALS AND METHODS

Medium

RPMI 1640 (Clinical Research Centre, Harrow, Middlesex) with HEPES (BDH) and 10 per cent v/v foetal calf serum (FCS) was used throughout. To prevent pinocytosis sodium azide (0·2 per cent w/v) was added to the medium used during the cell labelling procedure.

Cell suspensions

Thymocytes. Pieces of thymus were taken from children during cardiac surgery and this procedure added no additional risk to that of the operation itself. The tissue was stored for not more than 24 h at 4° in Eagle's minimum essential medium (Clinical Research Centre, Harrow, Middlesex) containing bicarbonate buffer, 3 per cent gelatine and a trace of deoxyribonuclease (Sigma). The thymus pieces were forced through a nylon sieve and filtered through washed cotton wool. The cells were washed once in medium and suspended to a known concentration of about 10^7/ml.

Peripheral blood lymphocytes (PBL). Immediately before each experiment 30 ml of blood was taken from one of two normal female donors and defibrinated by gentle stirring. Phagocytic cells were removed by a magnet after incubation with carbonyl iron (powder type S.F., G.A.F. Ltd, Manchester). One volume of freshly prepared 3 per cent gelatine (BRMFRA Ltd, Leatherhead, Surrey) was added to 2 volumes of blood and the erythrocytes were left to sediment for 45 min at 37°. Cells from the leucocyte-rich supernatant were washed and resuspended in medium to a known concentration of about 10^7/ml.

The viabilities of all cell suspensions, estimated by trypan blue exclusion, were above 90 per cent.

Antisera

The types of antisera used, their mode of preparation and specificities are summarized in Table 1. All antisera and normal control sera were absorbed twice with a half volume of packed human erythrocytes pooled from A, B and O donors. No antibodies to non-Ig serum proteins could be detected in Ouchterlony plates. Immediately before cell labelling all sera were centrifuged at 100,000 *g* for 20 min or 1 h.

Iodination of antiglobulins

IgG was isolated from the antiglobulin sera (listed in Table 1) by elution from DEAE with phosphate buffer (0·015 M, pH 7·4) and samples were iodinated using chloramine-T (Greenwood, Hunter and Glover, 1963). IgG and carrier-free ^{125}I (500 μCi in 20 μl) were mixed with one drop of phosphate buffer (0·5 M, pH 7·4). Freshly dissolved chloramine-T (0·2 mg in 0·1 ml phosphate buffer, 0·05 M, pH 7·4) was added, followed 1 min later by sodium metabisulphite (1·5 mg in 0·15 ml of distilled water) and one drop of 0·5 M KI. The reaction mixture was applied to a column of Sephadex G-100 and the iodinated IgG was collected into FCS. Recovery of ^{125}I-labelled IgG was assumed to be 100 per cent for estimation of specific activities. The efficiency of labelling ranged from 68 to 90 per cent in five different preparations with a mean of 80 per cent and the specific activity of the ^{125}I-labelled antiglobulins was between 7 and 9 μCi/μg. They were stored as solutions of 5 μg/ml in medium at $-30°$ and used for up to 1 month later.

Procedure for labelling lymphocytes (Wilson and Nossal, 1971)

Cell suspensions were dispensed to give pellets of $2·5 \times 10^6$ cells in each tube. Cells were resuspended in 0·2 ml volumes of antisera or control sera which had been diluted in medium containing 0·2 per cent sodium azide. After 30 min at 4° they were washed four times in 1 ml volumes of medium with sodium azide and resuspended in 0·2 ml volumes of ^{125}I-labelled antiglobulin containing 2–5 μg of the iodinated protein. After 30 min at 4° the cells were again washed four times and finally smeared on to microscope slides. The smears were fixed in methanol, washed in tap water and photographic film (Kodak

AR 10 stripping film) was applied. Replicate slides prepared from each tube were developed at various times ranging from 5 days to 10 weeks later. They were then stained with Giemsa and radioactively labelled cells were counted under a light microscope.

Criteria for estimation of labelled cells

In all experiments at least two replicate smears exposed for the same length of time were counted 'blind'. For PBL 250 cells and for thymocytes 500 cells were counted on each smear; 500 and 1000 cells per tube respectively. Cells with more than 100 grains were designated heavily labelled, those with 50–100 grains medium labelled, and those with more than 10 or 20 grains lightly labelled. In most experiments background labelling was low, from 0–5 grains per cell. Estimates of percentage labelled cells included all positive cells, heavy, medium and lightly labelled.

Controls

In each experiment, normal fowl and rabbit serum were included for specificity of antisera binding. For a positive control anti-thymocyte serum was included. In one experiment with thymocytes (from the 5-year-old child, Table 3) 0·15-mg amounts of purified human IgG were added to 1·0-ml volumes of the anti-F(ab′)$_2$ diluted 1:25 and 1:50 before cell labelling.

RESULTS

Peripheral blood lymphocytes

Although PBL were tested with at least three dilutions of all the antisera listed in Table 1, it was not possible to use all of these with each preparation of PBL, nor was the same batch of labelled anti-globulin used throughout. The results summarized in Table 2, therefore, are derived from six different experiments, each of which included other antisera and/or dilutions and the appropriate controls of normal serum. Exposure times for autoradiography were 5–7 days or in one case 3 weeks.

Anti-L chain antisera (rabbit and fowl pooled anti-κ and λ) at similar dilutions (1:25 and 1:50) labelled about the same percentage of PBL (8–11 per cent) and rabbit anti-IgG gave a similar result. The fowl anti-F(ab′)$_2$ however labelled 5–10 per cent more PBL at these dilutions and after L chain absorption the remaining anti-Fd antibodies at 1:25 labelled the same high percentage of cells. At higher dilutions (1:80 and 1:100) the percentage labelled with both anti-F(ab′)$_2$ and anti-Fd was similar to that of anti-L chain sera. The intensity of labelling, i.e. the proportion of cells with medium label within the positives was less with reagents made in rabbits than in fowls and no heavily labelled cells were seen with rabbit reagents.

Reagents for which results are not shown in Table 2 include sheep anti-IgM before and after absorption with IgG and IgA (4 and 5, Table 1) and fowl anti-κ IgG and anti-λ IgG myelomas (6 and 8, Table 1) each of which was tested before and after absorption with κ and λ Bence–Jones proteins respectively. The anti-IgM gave 7 per cent and the anti-μ gave 4 per cent of light PBL labelling. After the anti-IgG myeloma sera had been absorbed with L chains (7 and 9, Table 1) they labelled only 1–2 per

Table 1. Preparation and specificities of antisera used in cell labelling experiments

Antiserum no.	Immunizing antigen (human)	Species immunized	Absorbing antigen	Specificity	Species of antiglobulin used
1	Polyclonal IgG	Rabbit	None	κ, λ, γ	Rat
2	κ Chain*	Rabbit	Not known	κ	Rat
3	λ Chain*	Rabbit	Not known	λ	Rat
4	Monoclonal IgM	Sheep	None	κ, μ	Pig
5	Monoclonal IgM	Sheep	IgG, IgA	μ	Pig
6	κ IgG myeloma	Fowl	None	κ, γ	Rabbit
7	κ IgG myeloma	Fowl	Mixture of κ BJ proteins	γ	Rabbit
8	λ IgG myeloma	Fowl	None	λ, γ	Rabbit
9	λ IgG myeloma	Fowl	Mixture of λ BJ proteins	γ	Rabbit
10	Polyclonal F(ab′)$_2$†	Fowl	None	κ, λ, VH, Cγ1	Rabbit
11	Polyclonal F(ab′)$_2$†	Fowl	Polyclonal L chains	VH, Cγ1	Rabbit

* Nordic batches 670 (κ) and 2-371 (λ) (Nordic Immunological Laboratories, The Netherlands).
† Serum 534 in Orlans and Pearce (1974).

Table 2. Percentage of peripheral lymphocytes radiolabelled after treatment with anti-human Ig sera of different specificities and the appropriate ^{125}I-labelled antiglobulin

Reagent	Dilution	Percentage labelled peripheral blood lymphocytes	
		Mean value	Range (no. of experiments)
Pooled rabbit anti-λ and anti-λ (2+3*)	1/25	8	5–12 (3)
Rabbit anti-IgG (1)	1/25	9	7·5–10 (2)
Pooled fowl anti-κ and anti-λ (6+8)	1/25–1/50	11	8·4–11 (3)
Fowl anti-F (ab′)$_2$ (10)	1/25	17	15–18 (2)
	1/50	18	15–21 (2)
	1/100	10	9–11 (2)
Fowl anti-Fd (11)	1/30	17	(1)
	1/40	12	9·5–13·5 (2)
	1/80	9	7–11 (2)

* For details of reagents see Table 1.

cent of PBL showing that their high anti-γ antibody content (demonstrable by precipitation in agar gel) contributed little to the PBL binding activity shown in Table 2. Soluble complexes would be present in these sera after absorption and the low percentage of labelled cells also indicates that complexed fowl IgG did not bind to Fc receptors on the PBL.

Results on PBL were obtained with reagents and dilutions which were also tested on thymocytes.

Thymocytes

Results of three experiments with thymuses from children aged 5 months to 5 years are shown in Table 3. Exposure times for autoradiography were 7 weeks (5 months, F) or 3 weeks (5 years, M and 5 month, F).

No anti-immunoglobulin antiserum other than fowl anti-F(ab′)$_2$ (10 in Table 1) and anti-Fd (11) labelled thymus lymphocytes. The mean percentage of labelled thymocytes obtained with anti-F(ab′)$_2$ diluted 1:25 or 1:50 was 19 per cent. Moreover, after 3 weeks exposure to film the number of grains on the positive cells was only slightly less than that seen on PBL treated with the same antiserum (Fig. 1). At higher dilutions of anti-F(ab′)$_2$ i.e. 1:100 which still gave substantial labelling of PBL (Table 2) the percentage of labelled thymocytes decreased sharply

Table 3. Percentage of labelled lymphocytes found in three thymuses after treatment with various fowl antisera or normal fowl serum, and with ^{125}I-labelled rabbit anti-fowl IgG

Reagent	Dilution	Percentage labelled lymphocytes in thymuses of three children aged:		
		5 months F	5 years M†	5 months M
Anti-F (ab′)$_2$ (10)*	1:25	16	24	22
	1:50	n.d.	21	13
Anti-Fd (11)	1:25	n.d.	7	6
	1:50	n.d.	n.d.	4
Pooled anti-κ and λ (6+8)	1:25	n.d.‡	1	2
Normal fowl serum	1:25	<1	<1	<1

* For details of reagents see Table 1.

† Thymocytes were used within 3 h after surgery.

‡ Rabbit anti-κ and anti-λ pooled sera (2+3) were tested at dilutions to 1:8 on cells from this thymus. Less than 2 per cent of cells were radiolabelled.

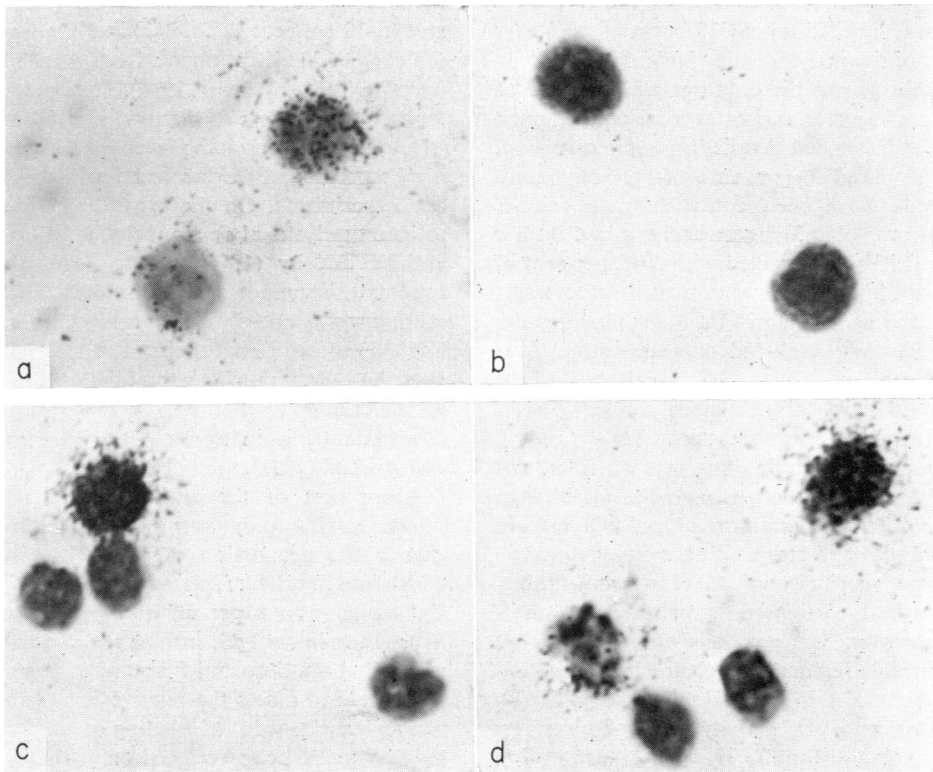

Figure 1. Autoradiographs of human lymphocytes after incubation with fowl anti-F $(ab')_2$ diluted 1:25. (a) and (b) Thymocytes from a 5-month-old female (Table 3). (a) One medium labelled cell and one classed as unlabelled because background was high. (b) Duplicate smear showing two unlabelled thymocytes. (c) and (d) Peripheral blood lymphocytes, two are heavily labelled and five unlabelled. (d) The lightly labelled cell contains carbonyl iron and these phagocytic cells were excluded from cell counts. (Magnification × 1575.)

to less than two. Also in contrast to results with PBL, the use of anti-Fd at 1:25 and 1:50 on thymocytes reduced the number of labelled cells to about one third of the value obtained with anti-$F(ab')_2$ at the same dilutions. The addition of human IgG to the anti-$F(ab')_2$ serum (see Methods) reduced the number of labelled cells to less than 1 per cent.

To see whether capping of the iodinated antiglobulin occurred in the absence of azide, in two experiments duplicate tubes without azide were included for each dilution of the fowl anti-$F(ab')_2$ serum. Cells from these tubes were smeared (a) immediately after the last cold wash and (b) 30 min later after incubation at room temperature. In each instance smears made from cell preparations without azide had very few labelled cells, corresponding to about one-fifth of the azide-containing controls and no convincing caps were seen.

DISCUSSION

The binding of an anti-human Ig antiserum (fowl anti-$F(ab')_2$) to human thymocytes implies the presence of Ig or of a material with structural similarities to Ig on the thymocyte membrane. Evidence that this antiserum is specific for Ig or Ig-like material is provided by the complete inhibition of binding to thymocytes by the addition of purified IgG (see Results section). No antibodies to non-Ig serum proteins, including β_2-microglobulin were detected in this antiserum in Ouchterlony plates (Orlans and Pearce, 1974).

We assume that Ig on thymocytes is synthesized by the cells themselves and not passively absorbed from blood or from the known one percent of B cells and plasma cells in the thymus (Goldstein, 1966; Hudson *et al.*, 1974) for two reasons. First,

only a subpopulation of cells from each of three thymuses was labelled (about 20 per cent) and over 90 per cent of thymocytes were viable. Secondly, the fowl anti-F(ab′)$_2$ was the only one which bound to thymocytes of several anti-Ig sera tested, although all the antisera labelled B cells in peripheral blood. Passively absorbed Ig or antigen–antibody complexes should have been detected by the anti-L chain antisera (Table 3); these antisera had both κ and λ specificities and reacted with 5–12 per cent of PBL but with less than 2 per cent of thymocytes.

The binding of fowl anti-F(ab′)$_2$ to Fc receptors on thymocytes was excluded because normal fowl serum and the fowl anti-L chain antisera did not bind. Furthermore, these latter antisera, after absorption with Bence–Jones proteins (7 and 9, Table 1), contained soluble complexes which did not bind to PBL nor did the fowl anti-F(ab′)$_2$ bind to thymocytes after the addition of purified IgG in amounts sufficient to form visible precipitates.

The broad specificity of the fowl anti-F(ab′)$_2$ appeared to be the essential factor for detection of Ig on thymocytes. The reactions of this antiserum with the variable regions of κ chains have been described (serum 534 in Orlans and Pearce, 1974). Evidence for anti-VH activity was obtained by testing several anti-F(ab′)$_2$ sera, after absorption with L chains, against a panel of monoclonal Ig of other classes. Serum 534 was chosen because it had very marked anti-VH activity, precipitating strongly with nine of fourteen monoclonal IgM proteins regardless of their light chain types. Fowls, perhaps because they are phylogenetically remote from mammals, produce antibodies consistently to V-region antigens when polyclonal human F(ab′)$_2$ is the immunogen; with monoclonal proteins they form mainly anti-C region and anti-idiotypic antibodies. For example, the fowl anti-κ and λ sera were raised against κ and λ IgG monoclonal proteins (6 and 8, Table 1) and contained antibodies to Cκ and Cλ and to Fc, but had little anti-V region activity. Thus, specificity predominantly for constant regions may explain why human thymocytes were not labelled by this fowl antiserum pool. Human thymocytes were not labelled by rabbit antisera specific for the variable region subgroups κI and κIII either (Grey, Colon, Solomon and McLaughlin, 1973) but the specificities of these rabbit antisera may have been highly restricted.

The percentage range of PBL labelled by the fowl anti-F(ab′)$_2$ (10–21 per cent) was larger than the B-cell population identified with the other anti-Ig sera (5–12 per cent in Table 2) but is still well within the range found by others: from 5 to 30 per cent (summarized by Warner, 1974). The broad specificity for Ig determinants of the fowl anti-F(ab′)$_2$ and its strong reactivity probably account for the relatively high percent of PBL labelled by this antiserum in our experiments. On the other hand, the antisera to intact polyclonal or monoclonal IgG with strong anti-Fc activity (1, 6 and 8, Table 1) labelled roughly the same percentage of PBL as those containing only anti-L chain antibodies (2 and 3, Table 1) showing that their anti-Fc activity did not contribute very much to the binding to PBL. This result, indicating that only a few PBL carry γFc determinants, is in agreement with Pernis, Forni and Amanti (1971).

Absorption of the anti-F(ab′)$_2$ serum with L chains leaving only anti-Fd specificities did not change the proportion of PBL (12–17 per cent) which was labelled. This suggests that VH and/or Cγ1 antigens are expressed on most B lymphocytes. Antisera to intact IgG, such as 1, 6 and 8 in Table 1, generally have little anti-Fd activity. It is, therefore, impossible to assess the relative importance of VH and Cγ1 antigens in the binding of the fowl anti-Fd reagent to PBL by comparison with results from other antisera.

With thymocytes, however, the anti-Fd serum labelled only one-third of the cells found positive with anti-F(ab′)$_2$. This suggests that L chain antigens are present on these cells despite the failure of anti-L chain sera to detect them. The large reduction in labelled cells found with fowl anti-F(ab′)$_2$ in the absence of azide (to about one-fifth of that in the presence of azide) indicates that endocytosis or shedding of Ig occurs very readily, even in the cold. According to Moroz and Hahn (1973) human thymus can synthesize Ig in short-term culture; this material was present in both membrane and cytoplasm and consisted of non-covalently linked heavy and light chains but could only be demonstrated when protease inhibitors were used throughout. If the thymocyte Ig is indeed very labile, as these two findings indicate, it may be necessary to have antibodies to multiple determinants or to both H and L chain antigens to provide anchorage for the radiolabelled portion of the sandwich.

We did not detect Ig on peripheral blood T cells however with the fowl anti-F(ab′)$_2$ serum. In one of

our experiments the exposure time for autoradiography of PBL was extended to 3 weeks, but the number of labelled cells was only slightly increased showing that circulating T cells were not labelled even after an exposure time when Ig is detected on 20 per cent of thymocytes. Detection of endogenously synthesized Ig on the thymocytes of young children but not on adult peripheral T cells resembles the situation in amphibians. In the toad the number of thymocytes carrying Ig of endogenous origin remains high during larval and early adult life, but decreases with age whilst no peripheral T cell Ig is detected at any stage (Du Pasquier, Weiss and Loor, 1972). Detection of Ig on circulating T cells, whether passively absorbed or endogenously synthesized, may require higher concentrations of antisera, antiglobulin with high specific activity, and the longer exposure times used on isolated T cell populations by others (Nossal *et al.*, 1972; Hunt and Williams, 1974).

An accurate comparison of the amount of Ig on peripheral B cells with the amount on thymocytes could not be made because the specific activity of the labelled antiglobulins was not known precisely. Nonetheless, the number of grains on thymocytes after 3 weeks exposure was only slightly less than on positive PBL after 7 days exposure, implying that similar amounts of Ig were present on both cell types. Marchalonis and Cone (1973) also found equal amounts of membrane bound Ig on thymocytes and B cells, but others do not endorse this (Warner, 1974). Prolonged exposure for 10 weeks increased the number of positive thymocytes and the number of grains per cell in our experiments, but also increased the number in controls with normal fowl serum so that the net percentage and intensity of specifically labelled thymocytes was unchanged.

Our results indicate that an immunoglobulin-like material is present on a subpopulation of thymocytes from young children. The size of this population could be larger than the 20 per cent found with serum 534 because this particular antiserum did not react with all heavy chains; it precipitated with two-thirds of the monoclonal μ chains tested. Nonetheless, anti-VH activity may be the essential bridge for binding of ^{125}I-labelled antiglobulins to thymocytes both because serum 534 showed strong anti-VH activity in gel and because other antisera which did not bind to thymocytes did not have either anti-VH activity or the broad anti-VL activity of serum 534. Alternatively, this fowl anti-F(ab')$_2$ cross-reacts with a substance on thymocytes with structural similarities to V region. β_2-Microglobulin has homology with IgG but primarily with the second and third constant domains, and also it is found on all cell types (Cunningham, 1974), and so it cannot be implicated here. Nonetheless, the presence on thymocytes of variable region structures which would include antibody-binding sites makes this material a candidate for the antigen recognition unit.

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NOTE ADDED IN PROOF

Recent studies (Binz, Kimura & Wigzell, 1975, *Scand. J. Immunol.* **4**, 413) have shown that T and B lymphocytes reactive against the same alloantigen have antigen-binding receptors with similar or even identical idiotypic determinants. The authors suggest that T and B cells use a common gene pool, particularly with regard to the heavy chain V region genes, to form their antigen binding receptors.

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