

## IMMUNOGLOBULIN D AS A LYMPHOCYTE RECEPTOR

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The function of immunoglobulin D (IgD) in the immune response is not yet clear; antibody activity in this immunoglobulin class has not been found as easily as in other classes and, so far, efforts to show a biological activity in IgD such as complement activation or reactivity with skin, mast cells, or neutrophils have given negative results (1, 2). By contrast IgD has been found to be present on the membrane of a variable proportion of peripheral blood lymphocytes in human adults (3) and, recently, on a much higher percentage of newborn (cord) blood lymphocytes (4).

The investigations to be reported here were performed to study the possible association of IgD with other immunoglobulin classes on the membrane of human lymphocytes and investigate whether IgD might be passively acquired as a cytophilic molecule. Our results show that IgD is usually associated with immunoglobulin M (IgM) on lymphocyte membranes and virtually eliminate the possibility that IgD is acquired by a cytophilic process. These findings provide support for the concept that IgD is a lymphocyte receptor.

### *Materials and Methods*

#### *Antisera and Fluorochrome Conjugates.*—

*Anti-IgD:* Two different antisera were used. One (R 39) was prepared by immunizing rabbits with D-myeloma protein isolated from a single serum by preparative starch-block electrophoresis. The animals received 5 mg of IgD emulsified in complete Freund's adjuvant subcutaneously and were boosted 1 mo later with 5 mg of the same protein in incomplete adjuvant; they were then bled a week later. The immunoglobulin G (IgG) fraction of the pooled rabbit antisera was isolated by DEAE-cellulose column chromatography and was conjugated with fluorescein isothiocyanate (FITC)<sup>1</sup> (Baltimore Biological Laboratories, Baltimore, Md.) by dialysis (5). The resulting conjugate had a fluorescein to protein molar (F/P) ratio of 2.5. Another aliquot was conjugated with tetramethylrhodamine isothiocyanate (TRITC) with the procedure of Cebra and Goldstein (6) as modified by Amante et al. (7). The conjugates were then made specific by absorption; this was performed by adding to 20 ml of the conjugate (0.5 mg of protein/ml), a mixture of 5 mg of IgG (Cohn fraction II, Mann Research Laboratories, New York), 2.5 mg of IgM prepared as indicated by Pernis et al. (8), and 1 ml of IgD-deficient serum. All the absorbents were insolubilized by ethyl chloroformate (9) and

<sup>1</sup> Abbreviations used in this paper: FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

were left in contact with the conjugate overnight at 4°C; the absorption was repeated twice. After absorption, the conjugates did not react with human IgG or IgM plasma cells obtained from the bone marrow from cases of multiple myeloma or Waldenström's macroglobulinemia. On the other hand, they gave a faint but definitive precipitin line when tested with a D-myeloma serum.

The other antiserum was prepared independently as indicated by Rowe et al. (4). The absorbed goat antiserum was conjugated with FITC, as previously described, and with TRITC (7). The conjugates were further absorbed with IgM-coated polyacrylamide beads (10) using IgM prepared from a case of Waldenström's macroglobulinemia. Conjugates prepared from both antisera gave similar results.

*Anti-IgM*: Again two different antisera were used. One was prepared by immunizing rabbits with IgM prepared from different cases of Waldenström's macroglobulinemia as indicated above. The immunization procedure as well as the preparation of the conjugates were the same as for the anti-IgD serum R 39. The absorption of the conjugates was also performed in the same way, but 3 mg of insolubilized IgD (prepared from D-myeloma serum as indicated above) was substituted for the 2.5 mg of IgM in each absorption. The anti-IgM conjugates were tested for specificity on human plasma cells from the bone marrow of IgG-myeloma patients, which did not stain, as well as in double staining with a specific anti-IgG on normal human spleen plasma cells, which showed no doubly stained cells except occasional double IgG and IgM producers.

The second anti-IgM serum was prepared by immunization of sheep with the IgM isolated from two cases of Waldenström's macroglobulinemia. Before conjugation, the antiserum was absorbed with glutaraldehyde-insolubilized human cord serum (11) so as to be specific for IgM by gel diffusion tests. Conjugation with FITC and TRITC was carried out as described for the second anti-IgG antiserum. The conjugates were further absorbed with a mixture of insolubilized IgM-deficient serum and of two insolubilized D-myeloma protein preparations (11). As an additional check on the specificity of these conjugates, a mixture was prepared of TRITC-labeled anti-IgD and FITC-labeled anti-IgM. Absorption of this mixture with insolubilized IgM removed the ability of the mixture to stain lymphocytes with fluorescein but left rhodamine unaffected. Absorption with insolubilized IgD had the reverse effect: fluorescein staining was unaltered; rhodamine staining became weak or absent. Conjugates prepared from both antisera gave similar results.

*Anti-κ and anti-λ*: One anti-κ and one anti-λ were prepared, conjugated with fluorochrome, and checked for specificity by immunofluorescence as previously described (12). A second pair consisting of a sheep anti-κ and a rabbit anti-λ were selected on the basis of their specificity and their reactivity both with free light chains and with intact immunoglobulins. In particular, the anti-λ was shown to precipitate several D-myeloma proteins in immunoelectrophoresis. The anti-κ was conjugated with fluorescein and the anti-λ with rhodamine as described above (4, 7).

Anti-immunoglobulin was prepared by immunization of rabbits with human Cohn fraction II using the schedule described above for anti-IgD R 39. The antiserum was conjugated by the dialysis method (5). Before use, all conjugates were ultracentrifuged at 100,000 g for 60 min to remove aggregates.

*Preparation of Lymphocytes, Staining for Surface Immunoglobulins, and Fluorescence Microscopy*.—The procedure followed at Basle for human peripheral blood lymphocytes (from cord blood or from adult blood obtained by venepuncture) was essentially the same as that described by Pernis et al. (8). When double staining for IgD and IgM was performed, 0.05 ml of each conjugate (one rhodamine labeled and one fluorescein labeled) was mixed and added to 0.05 ml of the cell suspension ( $10\text{--}20 \times 10^6$  cells/ml).

At Lausanne, lymphocytes were separated and stained as described previously (4). The preparations were viewed using a Zeiss Universal microscope (Carl Zeiss, Inc., New York) equipped with ultraviolet epiillumination and using  $\times 40$  or  $\times 63$  objectives and  $\times 10$  eye-

pieces. The filter system permitted separate identification of fluorescence due to fluorescein and rhodamine. Cells were usually viewed after fixation of smears but similar results were obtained on cells in suspension. Cyto-centrifuged preparations showed up to 30% monocytes in some preparations. Removal of monocytes by treatment with carbonyl iron and magnets did not affect the results. Most experiments of double staining were performed using mixtures of conjugates, after it had been shown that sequential treatment with each conjugate separately gave the same results. Faint staining of large mononuclear cells was seen in some experiments in both laboratories; these cells were not counted as immunoglobulin-positive lymphocytes.

*Anti-Immunoglobulin-Induced Redistribution (Capping) of Surface Immunoglobulins.*—

*Separate capping of IgD and IgM:* In these experiments, the lymphocytes were stained first with anti-IgD or anti-IgM conjugated with fluorescein. After staining, the cells were kept for 30 min at 37°C in Hanks' fluid with 10% fetal calf serum to allow capping; they were then restained with a rhodamine conjugate either of the same antiserum used for capping or of an antiserum directed against the other immunoglobulin class. The second staining was under noncapping conditions, that is in the cold (4°C) and in the presence of 2 mM NaN<sub>3</sub>. Thus in a typical experiment, the lymphocytes were first treated with fluorescein-conjugated anti-IgD and allowed to form green caps and then divided into two aliquots, one restained in "non-capping" conditions with rhodamine-conjugated anti-IgD (a check for the completeness of IgD capping) and the other restained with rhodamine-conjugated anti-IgM.

*Capping by anti-light chain antiserum:* The capping was induced as indicated above with nonconjugated anti- $\kappa$  and was followed by staining of the cells (under noncapping conditions) with a mixture of anti-IgD conjugated with rhodamine and anti-IgM conjugated with fluorescein.

*Capping and resynthesis:* The capping was induced with a variety of fluorescein-conjugated antisera after the procedure indicated above; the cells were then maintained for 14–20 h in tissue culture medium (RPMI) with 10% fetal calf serum at 37°C to allow the resynthesis of surface immunoglobulins (13). The newly synthesized immunoglobulins were detected by restaining with rhodamine-conjugated antisera. These experiments were carried out under sterile conditions.

## RESULTS

The percentage of lymphocytes positive for IgD and IgM and the percentage staining for both classes are shown in Table I. Similar results were obtained using the different anti-IgD and anti-IgM conjugates prepared in both laboratories. The mean value for IgD-positive cells in newborns was 14.6% and in adults 5.3%. A wide range of values was found in adults; repeated observations on the same individual showed that the values remained similar over a period of several months.

Most of the cells carrying IgD also carried IgM and vice versa. After double staining of cells from adults with anti-IgD and anti-IgM conjugates, an average of 75% of the IgD-positive cells were found to be IgM positive, and an average of 82% of the IgM-positive cells were found to be IgD positive. Similar higher values were obtained for the lymphocytes of the newborn. As these percentages show, a minority of IgD-positive cells clearly lacked demonstrable IgM and a minority of IgM-positive cells lacked IgD. There was considerable variation among different lymphocytes in the brightness of their staining for IgD and for IgM, suggesting that individual positive cells differed in the amount of each of these immunoglobulins on their membranes. Double-labeled cells varied widely

TABLE I  
*IgD and IgM on Peripheral Blood Lymphocytes*

Individuals	IgD	Percent positive cells*		
		IgM	IgD-M†	IgM-D‡
			%	
Newborn	12	18	86	82
"	12	9	86	91
Adult Ro	7 (5-8,10)	6 (6-6,3)	91 (90-93,3)	93 (91-96,3)
" Hu	3 (2-4,5)	6 (5-7,5)	67 (55-80,2)	60 (55-65,2)
" Zi	11 (10-12,4)	7	74	93
" Pf	2	2	70	82
Groups			Percent positive cells¶	
Newborns**	14.6 (7-26,14)	13 (9-18,2)	86 (86-86,2)	86 (82-91,2)
Adults	5.3 (2-17,12)	5.6 (2-7,5)	75.5 (67-91,4)	82 (60-93,4)

\* When the same individual was examined on different occasions, the range and the number of examinations are indicated in parentheses. For IgD and IgM, at least 500 cells were usually counted.

† The percent of cells initially identified as IgD-positive cells that were found also to be IgM positive. At least 100 cells were counted.

‡ The percent of cells initially identified as IgM-positive cells that were found to be IgD positive. At least 100 cells were counted.

|| Including the six individuals shown in this table.

¶ Mean for the group, with range and number of individuals in parentheses.

\*\* Includes previously published values (4).

in the intensity of staining with each fluorochrome, implying that the ratios of the amounts of each immunoglobulin differed among cells.

Further evidence that two classes of immunoglobulins were present on the membrane of the same cell was obtained from experiments involving independent capping. Lymphocytes incubated with fluorescein-conjugated anti-IgD at 0°C and in the presence of sodium azide showed fluorescence distributed in a granular pattern over the entire cell membrane. After incubation of these cells at 37°C for 30 min in the absence of azide, fluorescence was usually in the form of a cap at one pole of the cell, as described previously by Taylor et al. (14). To assess independent capping of the two classes, cells of two newborns were incubated with fluorescein-labeled anti-IgD under capping conditions and then exposed to rhodamine-labeled anti-IgM. Cells usually showed a green cap of fluorescence and a diffuse red granular staining due to rhodamine. Thus capping of IgD induced by the anti-IgD conjugate did not induce capping of IgM. If rhodamine-labeled anti-IgD were substituted for the rhodamine-labeled anti-IgM, the same cap showed both green and red fluorescence with no staining outside the cap. Similarly independent capping was also observed if the initial conjugate used for capping was fluorescein-labeled anti-IgM. In this case, rhodamine-labeled anti-IgD showed a diffuse staining. On the other hand, re-

staining with rhodamine-labeled anti-IgM gave caps with both green and red fluorescence and no diffuse staining.

In view of the presence of two heavy chain classes on the same cell, experiments were carried out to investigate whether both types of light chains could also be detected. When cells were exposed to a mixture of fluorescein-labeled anti- $\kappa$  and rhodamine-labeled anti- $\lambda$ , no double staining was seen in accordance with previous findings (15). In further experiments, cells were exposed to mixtures of conjugates of anti-IgD and anti-light chain labeled with different fluorochromes (Table II). Of the IgD-positive cells, an average of 55% appeared positive for  $\kappa$  and 41% positive for  $\lambda$ .

TABLE II  
*IgD,  $\kappa$ , and  $\lambda$  on Peripheral Blood Lymphocytes*

Individuals	Percent positive cells*			
	$\kappa$	$\lambda$	IgD- $\kappa$ †	IgD- $\lambda$ ‡
			%	
Ro	12 (5-18,3)	6 (3-9,3)	50 (47-54,2)	42 (39-46,2)
Zi	11 (6-16,2)	13 (10-16,2)	52 (50-54,2)	46 (44-48,2)
Hu	7	2	67	35
Pf	5	1	69	32
To	8	6	48	50
Newborn	6	7	45	38
"	3	4	55	43
Mean	7.4	5.6	55	41

\* See footnote \* of Table I. For  $\kappa$  and  $\lambda$ , 500 cells were usually counted. For IgD- $\kappa$  and IgD- $\lambda$ , 100 positive cells were counted.

† The percent of cells initially identified as IgD positive that were found also to be  $\kappa$ -positive.

‡ The percent of cells initially identified as IgD positive that were found to be also  $\lambda$ -positive.

Cells were also exposed to unlabeled anti- $\kappa$  under capping conditions. Subsequent exposure to a mixture of rhodamine-labeled anti-IgD and fluorescein-labeled anti-IgM showed capping of some cells. If capping was present, it was coincident and complete for both immunoglobulin classes. These findings showed that the IgD of one cell was of one light chain type only (with a slight preponderance of IgD- $\kappa$  cells) and that the light chain type of IgD and IgM on the same cell was identical. They also indicated that light chains, of either  $\kappa$ - or  $\lambda$ -type, could almost invariably be detected on IgD-positive cells. Finally, they provided direct evidence that the molecule stained by the anti-IgD was in fact immunoglobulin since it could be capped by anti- $\kappa$  chain antiserum.

It has been previously observed that after exposure of lymphocytes to anti-immunoglobulin conjugate incubation at 37°C causes capping followed by dis-

appearance of fluorescence due to endocytosis or shedding. After prolonged incubation, reexposure to fluorescent conjugate demonstrates a diffuse distribution of resynthesized immunoglobulin on the cell membrane (13). Similar findings were obtained for IgD. Lymphocytes of cord blood selected to show a high percentage of IgD-positive cells were tested. Exposure to fluorescein-conjugated anti-IgD or anti-immunoglobulin and incubation resulted in the formation of green caps within 1 h, as described previously. When the cells were restained with rhodamine-conjugated anti-IgD after 16–20 h incubation, a proportion of cells showed rhodamine staining in a diffuse distribution, but no cells showed any residual fluorescein stain. Thus there was evidence of reappearance of IgD on the membrane. Under similar conditions, IgM reappeared more rapidly; in one experiment, the number of IgM-positive cells was fully restored after 14 h incubation.

#### DISCUSSION

The high proportion of human peripheral blood lymphocytes carrying IgD on their membrane is in striking contrast with the very low concentration of IgD in the serum. The most extreme case is seen in cord blood where very many B lymphocytes have membrane IgD, whereas IgD cannot usually be detected, even by highly sensitive methods, in the serum (4). Even in adults, the percentage of IgD-carrying lymphocytes, although variable in different individuals, is always high in proportion to the IgD concentration on the serum (3).

Before considering the possible biological implications of these findings, two possibilities require elimination: (a) that reaction of human lymphocytes with anti-IgD antisera may have been due to cross-reactivity of the conjugated antisera with nonimmunoglobulin components of the lymphocyte membrane or with other immunoglobulin classes (notably IgM). This is ruled out by the following: (i) Anti-immunoglobulin and anti-light chain antisera capped all the determinants reacting with anti-IgD; this eliminates the possibility that the anti-IgD reacted with nonimmunoglobulin components of the cell membrane. (ii) There was independent movement of IgD and IgM on the lymphocyte membrane. Thus the staining given by the anti-IgD could not be due to some form of reactivity with IgM, since molecules reactive with anti-IgD did not react with anti-IgM and vice versa. (b) That IgD is not an active product of the lymphocytes that carry it but rather that it is passively absorbed by the cells as a cytophilic molecule. This possibility is also ruled out by two facts: (i) that cells from which IgD has been removed by exposure to antisera showed reappearance of IgD on their membrane during incubation in IgD-free medium; (ii) that lymphocytes that carry IgD (and IgM) carry only one type of light chain, i.e., either  $\kappa$  or  $\lambda$ . Passively adsorbed IgD would not be expected to be of one light chain type only, nor restricted to the same light chain type as membrane IgM. This observation was considered as crucial and was established

not only by double staining of the lymphocytes with anti-IgD and anti- $\kappa$  or anti- $\lambda$ , but also by showing that after treatment with anti- $\kappa$  antiserum a proportion of IgD-positive cells showed complete capping both of IgD and IgM. We therefore conclude that the IgD of the lymphocyte membrane is the active product of the cell that carries it and that IgD fulfills in this respect an obligatory requirement for a lymphocyte antigen receptor.

In the light of these findings, we propose the hypothesis that the thus far elusive biological function of IgD is that of an antigen receptor on the lymphocyte membrane. Other known features of IgD are consistent with this view. IgD is secreted only in small amounts and by very rare plasma cells (12), in striking contrast with the high proportion of lymphocytes having IgD on their membrane. The biological properties important for the effector roles of secreted immunoglobulins, such as complement activation, skin fixation, concentration in secretions, and placental transfer, have not been found in the IgD class (4).

The finding that both IgD and IgM are simultaneously present in a high proportion of lymphocytes is remarkable and does not follow the pattern of previous reports concerning other immunoglobulins where restriction to one class is the general rule (16). We consider three possible implications of this finding: (a) The combining sites of IgD and IgM receptors differ. This would probably require the same cell simultaneously to express two different  $V_H$  region genes and to link their products separately to those of two different  $C_H$  region genes. We regard this as unlikely. It seems more probable that both receptors have the same combining site, consistent with the presence of light chains of one type only. (b) The class of the receptor determines the signal to the cell. For example, tolerance and induction may be dependent on the heavy chain class of the receptor. (c) IgD constitutes the first antigen receptor. The appearance of IgM (potentially a secreted immunoglobulin) as a receptor indicates a step in differentiation towards a plasma cell. Thus one cell line may shift synthesis from one heavy chain class to another, the sequence being  $\delta \rightarrow \mu \rightarrow \gamma$  with  $\delta$  and  $\mu$  showing overlap. We favor explanations based on the second and the third possibilities, which are not in themselves mutually exclusive. In any event, we consider that the simultaneous presence of two receptors on the same cell indicates a remarkable genetic event of fundamental significance for immune responsiveness.

#### SUMMARY

A large proportion of the human peripheral blood lymphocytes of adults and newborns having IgD were found also to have IgM on their membranes and vice versa. A few lymphocytes had one of these classes only. IgD and IgM could be capped independently on the same cell. The possibility that IgD was acquired by a cytophilic process was excluded by the finding that IgD-bearing cells were of one light chain type only, and by the direct demonstration of re-appearance of IgD on the lymphocyte membrane during incubation in an IgD-

free culture medium. On the basis of these findings, it is proposed that IgD functions as a lymphocyte antigen receptor.

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