

# BIOSYNTHESIS OF LYMPHOCYTE SURFACE IgD IN THE MOUSE<sup>1</sup>

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The synthesis of IgD was studied in mouse spleen cells by using [<sup>35</sup>S]methionine labeling followed by immunoprecipitation with monoclonal antibody and two-dimensional gel electrophoresis. After a 15-min pulse of [<sup>35</sup>S]methionine, a relatively basic form (IgD1) of apparent m.w. 59,000 was precipitated. Conversion into more acidic forms of m.w. 63 to 72,000 (IgD2) took place during a chase period of several hours. The acidic form was identical in mobility to that of IgD labeled by surface radioiodination, and was almost completely removed by treatment of intact cells with pronase.

Neuraminidase treatment of the surface form (IgD2) produced a form resembling IgD1 in charge, but with no detectable change in m.w. Treatment of IgD1 with endoglycosidase H resulted in a form with an apparent m.w. of 50,000, whereas IgD2 was resistant to this enzyme. Both IgD1 and IgD2 bound to lentil lectin, whereas only IgD2 bound to *Ricinus communis* hemagglutinin, which binds to terminal galactose residues.

These results indicate that IgD is synthesized as an incompletely glycosylated precursor possessing "high mannose" type oligosaccharide moieties, and passes relatively slowly through the cell. Shortly before surface appearance, galactose and sialic acid are added.

No specific association with any other labeled protein was observed, and any IgD secretion was below the limits of detectability.

Most of the current knowledge of the biosynthesis and processing of cell surface glycoproteins has derived from studies of viral products made in infected cells (1, 2). Since viral glycoproteins are made by using host synthetic apparatus, one may expect that nonviral cell surface glycoproteins will follow similar pathways. This has recently been demonstrated to be the case for the major human erythrocyte membrane protein glycoporphin, synthesized in a leukemia line (3).

The lymphocyte surface immunoglobulins are also among the most intensively studied and best characterized of cell surface proteins. They act as antigen receptors, and differences in surface immunoglobulin phenotype at various stages of B lymphocyte differentiation seem to be associated with differ-

ences in functional capacity regarding tolerance, immunity, and memory (4).

The major lymphocyte surface immunoglobulin classes are IgM and IgD, and most mature but virgin or nonmemory B lymphocytes possess both these isotypes (5). On the other hand, immature B cells and late memory B cells lack IgD (6). Furthermore, memory cells for low affinity clones possess IgD whereas memory cells for high affinity clones lack IgD (7).

Nearly all biochemical studies of lymphocyte surface IgD have involved lactoperoxidase-catalyzed cell surface radioiodination, and there is little information in the literature concerning its biosynthesis, processing, kinetics of passage through the cell, or possible association with other proteins (8). This lack is somewhat surprising. Unlike all other immunoglobulins, IgD is rarely secreted and is almost exclusively found on the cell surface. It therefore represents an ideal model for receptor biosynthesis, since results are unlikely to be complicated by concomitant secretory forms (9, 10).

In this paper, we describe the biosynthesis of lymphocyte surface IgD by normal murine spleen cells, and show that many features are similar to the biosynthesis of glycoporphin. IgD is shown to be synthesized as an incompletely glycosylated cytoplasmic precursor, which is distinct from surface IgD in both charge and size. Transit through the cell is relatively slow, but final processing and surface appearance occur within a short time of each other. No specific association with another labeled protein could be demonstrated, and any IgD secretion was below the limits of detectability.

## MATERIALS AND METHODS

**Biosynthetic labeling and membrane solubilization.** Spleen cells from 8-week-old-male BALB/c mice were prepared by gently pressing the spleen between the frosted ends of two glass slides. Cells were washed twice, and cultured at 37°C by using  $2.5 \times 10^7$  cells/ml in 2-ml methionine-free RPMI 1640 medium containing 15% dialyzed fetal calf serum and 1.0 mCi [<sup>35</sup>S]-methionine (800 Ci/mole; Amersham Corp., Arlington Heights, Ill.). After culture, cells were washed in methionine-containing RPMI 1640 and lysed in 0.5% Nonidet-P40 (Particle Data, Elmhurst, Ill.) for 30 min at 4°C. Nuclei were removed by centrifugation at  $5,000 \times G$  for 10 min. Kinetic experiments showed that the uptake of [<sup>35</sup>S] into trichloroacetic acid- (TCA) precipitable material increased linearly over a 6-hr period.

**Immunoprecipitation and two-dimensional gel analysis.** The detergent lysate was "precleared" by addition of 50  $\mu$ l of a 10% (v/v) suspension of heat-killed and fixed staphylococci (11) followed by centrifugation at  $5,000 \times G$  for 5 min. To the extract was added an excess of antibody (25  $\mu$ g of the IgG fraction of rabbit anti-mouse Fab; 2  $\mu$ l rabbit anti-mouse  $\mu$ ; 2  $\mu$ l rabbit anti-mouse  $\delta$ ). IgD was bound by 400  $\mu$ l culture supernatant of hybridoma 10-4 monoclonal anti-IgD allotype (anti-Igh-5a, Reference 12) containing approximately 10  $\mu$ g antibody.

Complexes were precipitated by addition of 50  $\mu$ l of a 10% (v/v)

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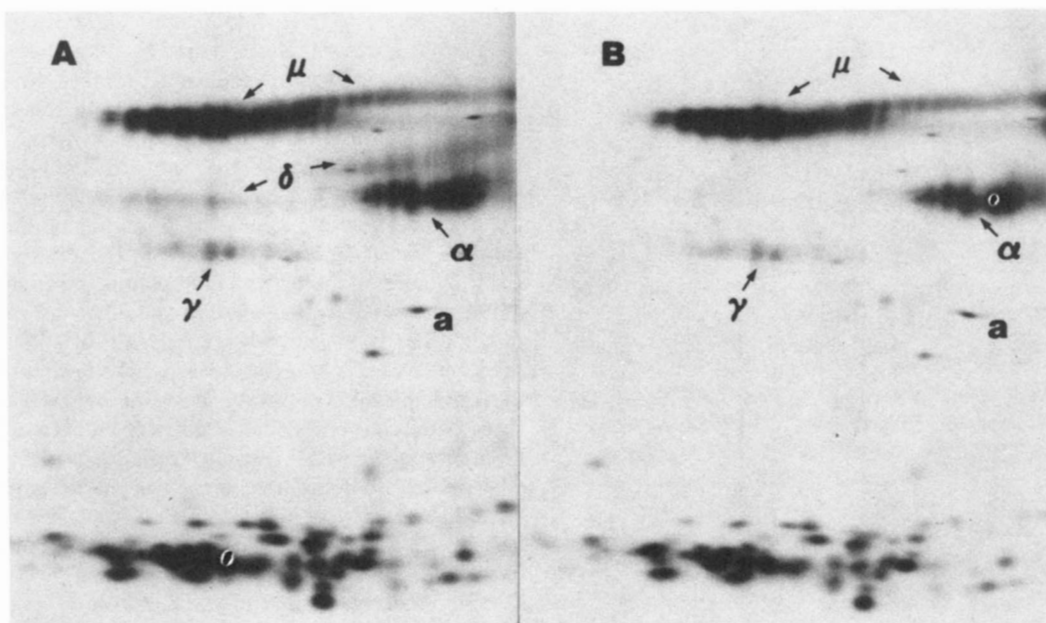


Figure 1. Two-dimensional gel electrophoresis of  $^{35}\text{S}$ -labeled immunoglobulin chains. Spleen cells from 8-week-old BALB/c mice were cultured for 4 hr in RPMI 1640 medium containing 1.0 mCi [ $^{35}\text{S}$ ]-methionine, washed, lysed in 0.5% Nonidet P40, and immunoglobulins precipitated by rabbit anti-Fab followed by staphylococci. Precipitates were reduced with mercaptoethanol and analyzed by two-dimensional gel electrophoresis. Acidic species lie to the right, basic species to the left. Actin is marked "a". B, IgD was removed by preprecipitation.

v) suspension of heat-killed and fixed staphylococci (11), followed by three washes in 0.05 M Tris, pH 8.3, containing 0.4 M NaCl and 0.5% Nonidet-P40. Pellets were finally resuspended in a buffer containing 8 M urea, 2% ampholines, 1% Nonidet-P40, 5% 2-mercaptoethanol, and the eluted proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis exactly as described (13). Typical exposure times for autoradiographs were 1 to 2 weeks.

**Pronase treatment.** After biosynthetic labeling for 2 hr, cells were washed and treated with 100  $\mu\text{g}/\text{ml}$  pronase (Calbiochem-Behring Corp., La Jolla, Calif.) at 37°C for 20 min, in phosphate-buffered saline (PBS; 164 mM  $\text{Na}^+$ , 4 mM  $\text{K}^+$ , 132 mM  $\text{Cl}^-$ , 20 mM  $(\text{PO}_4)^{3-}$ , pH 7.4). They were then washed once in RPMI 1640 containing 15% fetal calf serum, and lysed in 0.5% Nonidet P40 as previously described. Cell viability was not decreased by pronase treatment.

**Neuraminidase and endoglycosidase H treatment.** Staphylococcal pellets after immunoprecipitation were treated with 0.01 unit/ml, 0.1 unit/ml or 1.0 unit/ml neuraminidase (Worthington Biochemical Corp., Freehold, N.J.) or 1.0 unit/ml endoglycosidase H (Miles Laboratories, Inc., Elkhart, Ind.) in 0.1 M NaCl, 0.05 M HEPES,<sup>3</sup> pH 6.5, for 1 hr at 20°C. They were then washed and the bound proteins eluted as above.

**Cell surface iodination.** Spleen cells from 8-week-old-BALB/c mice were incubated for 5 min at 20°C in 0.168 M  $\text{NH}_4\text{Cl}$  to remove red cells. Dead cells were removed by centrifugation at 2,000  $\times$  G on a cushion of Isopaque/Ficoll, density 1.09, followed by three washes on PBS. Viability was always >97% as assessed by staining with acridine orange and ethidium bromide (14).

Cells were radioiodinated by a modification of the lactoperoxidase technique. To  $5 \times 10^7$  cells in 200  $\mu\text{l}$  PBS were added 1.0 mCi [ $^{125}\text{I}$ ] (Amersham Corp., Arlington Heights, Ill.). 10  $\mu\text{g}$  lactoperoxidase (B grade, Calbiochem-Behring Corp.) and successive 10- $\mu\text{l}$  pulses of  $\text{H}_2\text{O}_2$  (0.3 mM, 1 mM, 3 mM, and 9 mM) at 1-min intervals, at 20°C. Cells were washed twice in PBS

and lysed in detergent as above. Typically 1 to 3% of the radioactivity was incorporated into trichloroacetic acid-precipitable material.

**Lectin binding.** Detergent lysates of labeled cells were passed over columns consisting of tuberculin syringes containing 200  $\mu\text{l}$  agarose beads coupled to lentil lectin (Pharmacia Fine Chemicals, Piscataway, N.J.) or *Ricinus communis* hemagglutinin (E. Y. Laboratories, San Mateo, Calif.). Columns were equilibrated with isotonic saline buffered by 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.4, containing 0.5% (v/v) Nonidet P40 and 1 mM each of  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ , and  $\text{Mn}^{++}$ . Specificity of binding was confirmed by inhibition with 0.1 M  $\alpha$ -methyl mannoside or galactose, respectively.

## RESULTS

**Size and charge relationships of IgM, IgG, IgA, and IgD heavy chains.** In preliminary experiments, splenic lymphocytes were cultured for 2 hr with [ $^{35}\text{S}$ ]methionine, washed, and lysed in the nonionic detergent Nonidet P40. Immunoglobulin was precipitated with rabbit anti-mouse Fab, reduced, and analyzed by two-dimensional gel electrophoresis. In all experiments, the first dimension consisted of nonequilibrium pH gradient electrophoresis, and the second dimension sodium dodecyl sulfate-(SDS) polyacrylamide gel electrophoresis (13).

Results are shown in Figure 1-A. Parallel precipitations using class-specific antisera allowed identification of all the heavy chain bands. By far the major classes synthesized were IgM and IgA, whereas IgG was much less prominent. The paucity of IgG is only partially explained by the fact that all extracts were "nrecleared" with staphylococci since a) examination of the "preclear" precipitation did not reveal large amounts of IgG, and b) IgG1, which is a major subclass, binds weakly, if at all, to staphylococcal protein A (15). The two remaining heavy chain bands were identified as  $\delta$ -chains, since they were specifically removed by preprecipitation with monoclonal anti-IgD (Fig. 1-B). It should be noted that  $\alpha$ -chains have very similar mobility to one of the  $\delta$ -bands.

<sup>3</sup> Abbreviations used in this paper: HEPES, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; RCA, *Ricinus communis* hemagglutinin-agarose; SDS, sodium dodecyl sulfate.

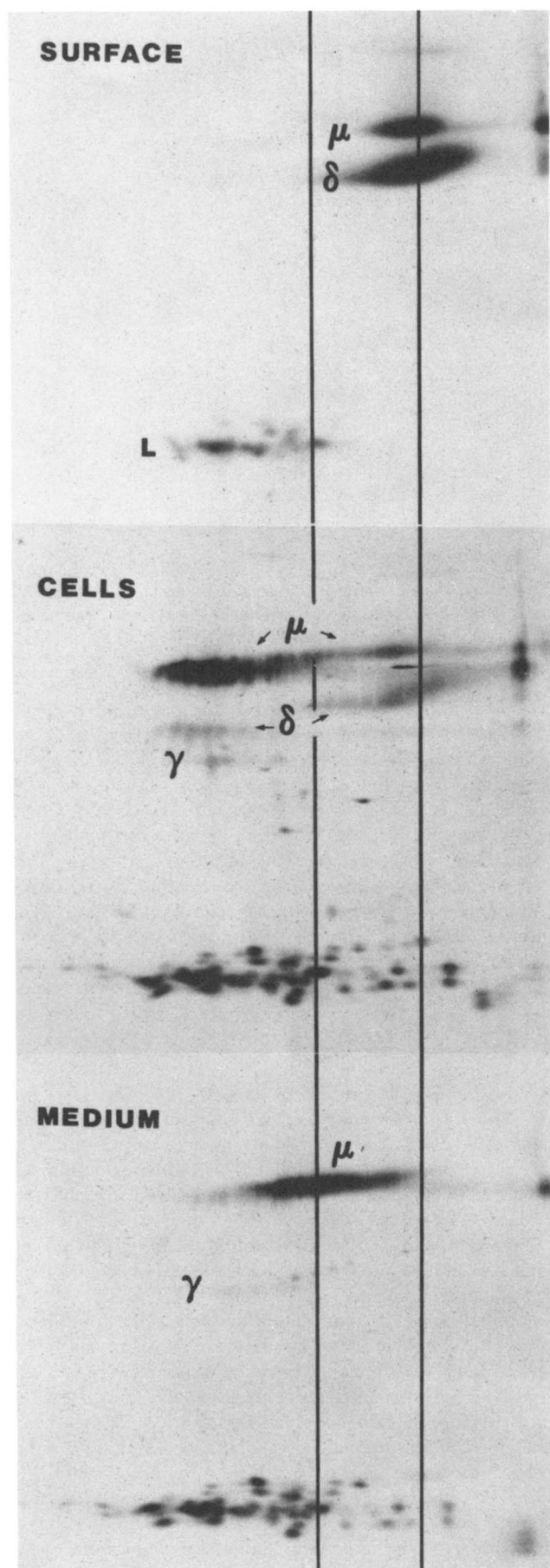


Figure 2. Comparison of surface cytoplasmic and secreted immunoglobulins. Spleen cells from 8-week old BALB/c mice were labeled with  $^{125}\text{I}$  by the lactoperoxidase technique (upper), or metabolically labeled

Three forms of IgM; two forms of IgD. Figure 2 shows the results of an experiment designed to show the size and charge relationships between cytoplasmic, surface, and secreted immunoglobulins. For concise presentation, the diagrams show immunoprecipitation with anti-Fab antibodies, although all bands were identified by parallel precipitation with chain-specific antisera. For clarity,  $\alpha$ -chains were removed by prior precipitation. The upper panel shows the analysis of immunoglobulins precipitated from detergent lysates of surface radioiodinated spleen cells. Only  $\mu$ -chains,  $\delta$ -chains, and light chains were seen. The central panel shows a parallel analysis of labeled immunoglobulins precipitated from the detergent lysate of [ $^{35}\text{S}$ ]methionine-labeled spleen cells, and the lower panel shows the immunoglobulins precipitated from the culture medium. By comparing the three panels, several conclusions may be drawn.

In agreement with previous work (16, 17), three forms of  $\mu$ -chains are seen. For convenience, we will designate them as  $\mu_1$ ,  $\mu_2$ , and  $\mu_3$ . Form  $\mu_1$  is present in the cytoplasm, and is the most basic form, whereas the more acidic  $\mu_2$  form is in the supernatant. Pulse-chase studies have shown that  $\mu_1$  is a precursor of  $\mu_2$  (18). The largest and most acidic form of  $\mu$  chain ( $\mu_3$ ) has a mobility in both dimensions that is identical to that of surface  $\mu$  labeled by surface radioiodination and is removed by protease treatment of intact cells (data not shown). Previous data in the literature indicate that surface  $\mu$ -chains migrate a little more slowly than secreted chains in SDS gels (16, 17, 19).

In contrast to  $\mu$ -chains, only two major forms of  $\delta$ -chains are seen. The smaller and more basic form ( $\delta_1$ ) has an apparent m.w. of 59,000 whereas the larger and more acidic forms ( $\delta_2$ ) have an apparent m.w. range of 63,000 to 72,000. The mobility of  $\delta_2$  is identical in both dimensions to that of surface radioiodinated  $\delta$ -chains (Fig. 2). There is no detectable secreted IgD.

*A cytoplasmic IgD precursor: kinetics of processing and surface appearance.* The existence of two forms of  $\delta$ -chains suggested a possible precursor-product relationship. A pulse-chase experiment was performed to test this hypothesis. Spleen cells were cultured with [ $^{35}\text{S}$ ]methionine for 15 min, and then a large excess of cold methionine was added. Aliquots of cells were removed and examined for IgD synthesis at the end of the pulse ( $t = 0$ ), after 15 min of chase ( $t = 15$  min), 1 hr of chase ( $t = 1$  hr), and 8 hr of chase ( $t = 8$  hr).

Results are shown in Figure 3. At  $t = 0$ , only the  $\delta_1$  form is seen. By  $t = 15$  min,  $\delta_2$  is visible, and at  $t = 1$  hr there is a substantial conversion into the  $\delta_2$  form. At 8 hr, nearly all the IgD is in the  $\delta_2$  form. Thus,  $\delta_1$  is a precursor of  $\delta_2$ .

The similarity in mobility in both dimensions of  $\delta_2$ -chains and the  $\delta$ -chains labeled by surface radioiodination suggests that some or all of the  $\delta_2$  form is present on the cell surface. This hypothesis was tested as follows. Spleen cells were cultured for 2 hr with [ $^{35}\text{S}$ ]methionine, at which time approximately equal amounts of labeled  $\delta_1$  and  $\delta_2$  were present. They were then harvested, washed, and treated with pronase (100  $\mu\text{g}/\text{ml}$  at  $37^\circ\text{C}$  for 20 min), washed again, lysed in Nonidet P40, and examined for IgD as previously described. Results are shown in Figure 4.

Treatment of cells with pronase removed the  $\delta_2$  form almost completely, but did not affect the  $\delta_1$  form. Since pronase is unable to penetrate intact lymphocytes, these results prove that most of the  $\delta_2$  form is present on the cell surface. The uptake of

with [ $^{35}\text{S}$ ]methionine (middle and lower) for 2 hr. After labeling, cells were lysed in 0.5% Nonidet P40 and immunoglobulins precipitated by rabbit anti-Fab followed by staphylococci. For clarity in presentation, IgA was preprecipitated with rabbit anti-IgA for the gel analyses in the middle and lower panels. Vertical lines indicate alignments of gels.

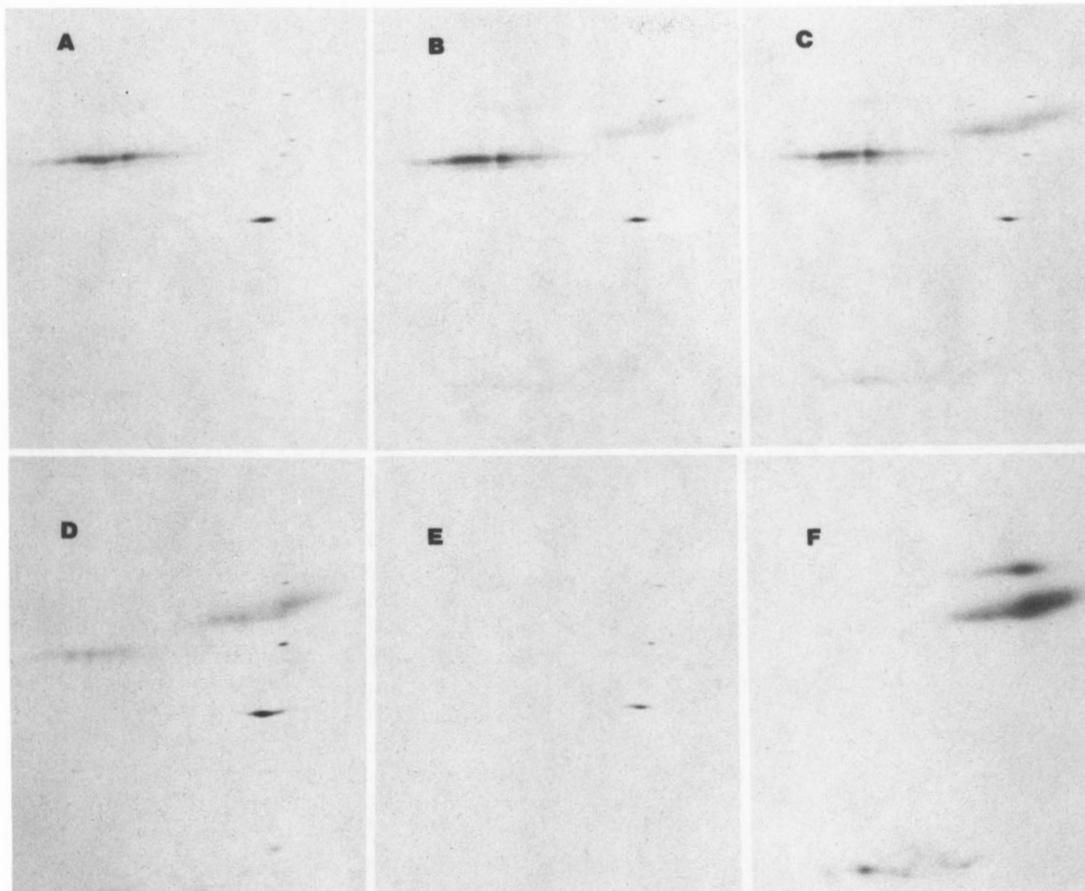


Figure 3. Pulse-chase analysis of IgD biosynthesis. Spleen cells from 8-week-old BALB/c mice were cultured for 15 min with [ $^{35}$ S]methionine, and then chased by addition of 1 mM unlabeled methionine ( $t = 0$ ). At various times, aliquots of cells were removed and lysed in 0.5% Nonidet P40, and IgD immunoprecipitated with 10-4 antibody. A,  $t = 0$ ; B,  $t = 15$  min; C,  $t = 1$  hr; D,  $t = 8$  hr; E, control precipitation (anti-Igh-5b); F, cell-surface immunoglobulins precipitated from surface-radioiodinated spleen cells by rabbit anti-Fab.

[ $^{35}$ S]methionine into TCA precipitable material increased linearly during the labeling period, and therefore newly synthesized IgD molecules are expected to move continuously from the  $\delta_1$  form to the  $\delta_2$  form. Thus, the results also indicate that once the final processing of IgD takes place, movement onto the cell surface occurs rapidly.

*The cytoplasmic precursor of IgD is incompletely glycosylated.* The precursor form of IgD is smaller and more basic than the surface form. Although it seemed very likely that transition from the precursor form of IgD involved the addition of neutral sugars and sialic acid, the uptake of labeled sugars by normal spleen cells was too low to allow direct testing of this hypothesis.

As an alternative to the use of labeled sugars, we chose to analyze the binding of IgD to lectins. Detergent lysates of cells labeled metabolically with [ $^{35}$ S]methionine, or cells that were surface labeled with [ $^{125}$ I], were passed over small columns of lentil lectin-agarose (specific for mannose, glucose, and related sugars) or *Ricinus communis* hemagglutinin-agarose (RCA; specific for terminal galactose), and the drop-through fraction was analyzed by immunoprecipitation.

All forms of all intact immunoglobulins studied (IgD1, IgD2, IgM1, IgM2, IgM3, IgA, and IgG) were totally depleted by passage over lentil lectin-agarose. However, a considerable amount of free light chains (estimated as 30% of the total synthesis) emerged in the drop-through fraction. In contrast, passage of radiolabeled material over RCA caused total depletion of IgD2 (surface IgD), but no detectable depletion of IgD1. Similarly, IgM1 did not bind detectably, whereas IgM2 and IgM3 were depleted quantitatively by RCA. IgG and IgA

showed partial depletion. These data are consistent with the notion that although the precursor forms of IgD and secretory IgM possess mannose residues, they lack terminal galactose residues. The end products (surface IgD and IgM, and secreted IgM) possess both mannose and terminal galactose.

The enzyme endoglycosidase H (20) attacks only the bond between the two *N*-acetyl glucosamine residues in the glucosamine-mannose containing core region of asparagine-linked oligosaccharides. It shows a strong preference for the "high mannose" type oligosaccharides, and removes all the carbohydrate from these chains except for the one *N*-acetyl glucosamine that remains attached to asparagine (1).

This enzyme was used to characterize further the carbohydrate structure of IgD1 and IgD2. Spleen cells were cultured with [ $^{35}$ S]-methionine for 2 hr, at which time approximately equal amounts of each form of IgD were present. Cells were lysed in detergent, IgD immunoprecipitated, and the staphylococcal pellets were treated with endoglycosidase H (Fig. 5). Enzyme treatment had no effect on the mobility of  $\delta_2$ -chains, but decreased the apparent m.w. of  $\delta_1$ -chains by approximately 10,000 daltons. These results suggest that processing of the oligosaccharides of IgD occurs in a similar manner to that of IgG oligosaccharides (21). The apparent m.w. of the endoglycosidase H treated  $\delta_1$ -chains was similar to that of  $\gamma$ -chains (Fig. 5).

The presence of sialic acid residues on lymphocyte surface IgM and IgD was examined by two-dimensional gel electrophoresis after neuraminidase treatment. Results are shown in Figure 6. (Gels were aligned by reference to light chains, which do

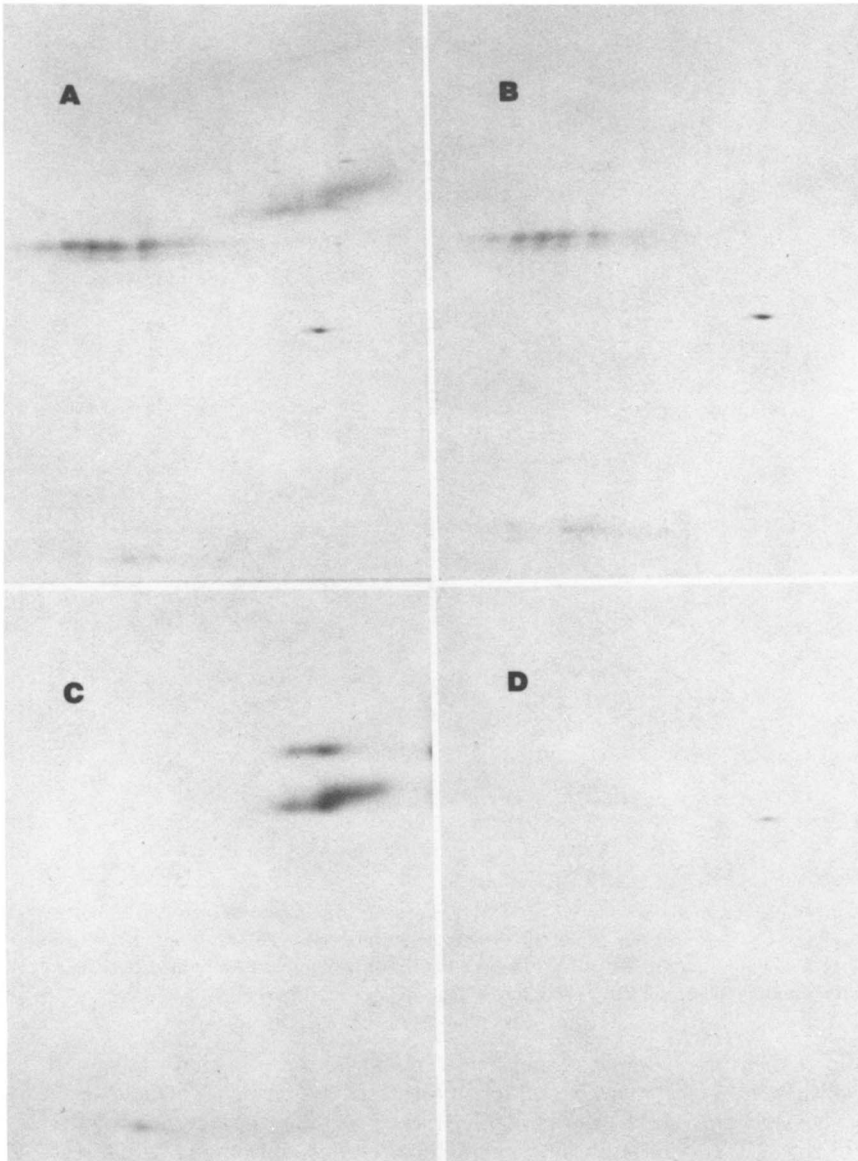


Figure 4. Proteolytic removal of cell surface IgD. Spleen cells from 8-week-old BALB/c mice were cultured for 2 hr with [ $^{35}$ S]methionine, washed, treated with pronase, and then lysed in 0.5% Nonidet P40. IgD was immunoprecipitated with 10-4 antibody and analyzed as in Figure 1. A, no pronase; B, pronase-treated cells; C, surface immunoglobulins labeled with  $^{125}$ I and immunoprecipitated with rabbit anti-Fab; D, control precipitation (anti-Igh-5b).

not possess carbohydrate, and staining of unlabeled marker proteins.)

Neuraminidase digestion of  $\delta_2$ -chains caused a marked shift in mobility toward the basic end of the gel. A similar, but less marked, shift of  $\mu_3$  occurred. Digestion of both  $\mu$ - and  $\delta$ -chains was complete in panel C, since a 10-fold higher enzyme concentration produced no further digestion (data not shown). The charge of the neuraminidase-treated surface  $\delta_2$  was similar to that of its precursor,  $\delta_1$ . On the other hand, neuraminidase-treated surface  $\mu_3$ -chains were still clearly more acidic than  $\mu_1$ -chains.

The charge differences between  $\delta_1$  and  $\delta_2$  can thus be explained by addition of sialic acid residues. We have not yet identified a precursor for the surface form of IgM. Although the data are consistent with the idea that surface IgM is synthesized from a precursor that is distinct from that of secretory IgM, the possibility that some sialic acid residues were not removed by neuraminidase cannot be ruled out.

*Lack of detectable association of IgD with other proteins.* It was of interest that no other labeled protein was immunoprecipitated together with IgD, except possibly actin, which was also present in the control precipitates. If IgD is associated with other proteins, failure to detect such a relationship could have

several reasons. First, the complex might be disrupted by detergent extraction. This possibility seems unlikely, but is hard to disprove. The associated protein might lack methionine. However, [ $^{35}$ S]-cystine and [ $^3$ H]-arginine labeling produced identical results (data not shown).

Yet another possibility is that an association with other proteins might be disrupted by the high salt buffers used in washing the staphylococci, the absence of divalent cations, or other ionic effects. In an attempt to address this question, detergent extraction and washes were carried out in HEPES-buffered isotonic KCl containing 1 mM  $\text{Ca}^{++}$  and 1 mM  $\text{Mg}^{++}$ , in order to mimic the intracellular environment more closely. Under these conditions, many other proteins were precipitated along with IgD by anti- $\delta$  (data not shown). However, all such spots were present to an equal extent in the control groups without antibody, and thus their precipitation was probably nonspecific.

#### DISCUSSION

Two-dimensional gel electrophoresis is capable of resolving the heavy chains of all the major immunoglobulin classes synthesized by murine spleen cells. The heavy chains of IgD, which is a very minor class in terms of total synthesis (2.7% of

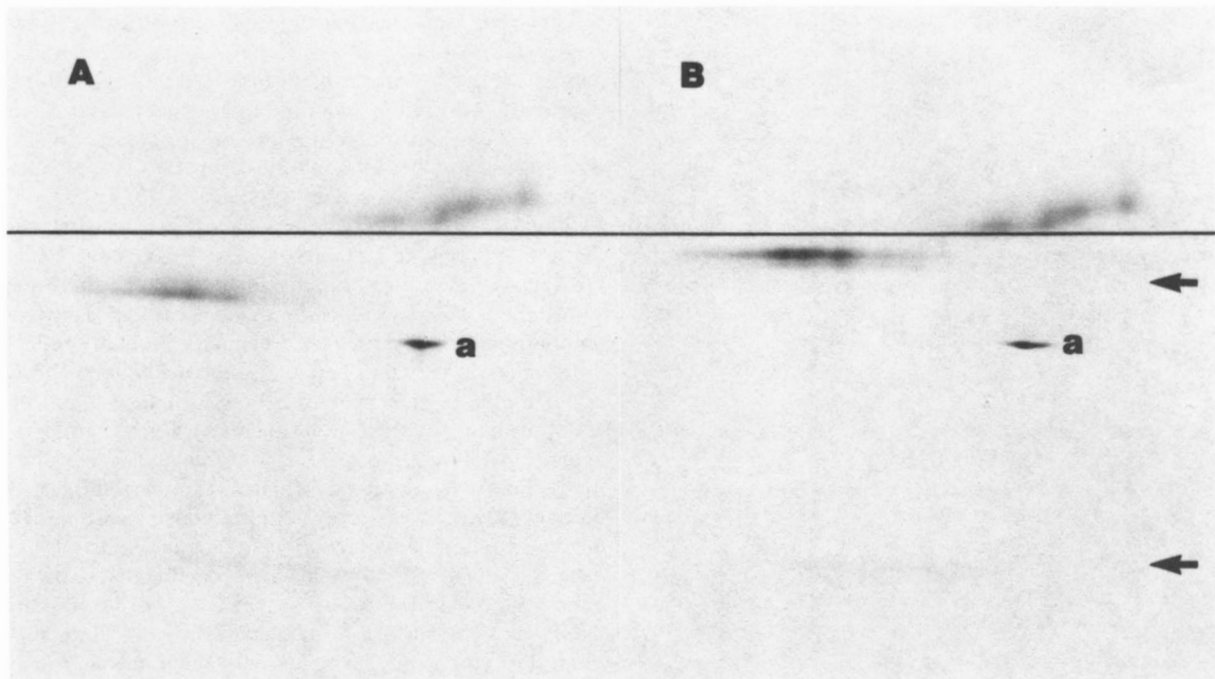


Figure 5. Treatment of IgD with endoglycosidase H. Spleen cells were cultured for 2 hr with [ $^{35}$ S]methionine, lysed in detergent, and IgD was immunoprecipitated with 10-4 monoclonal anti-Igh-5a and staphylococci. Pellets were treated with endoglycosidase H (A) or buffer alone (B) before analysis. Arrows indicate mobility of  $\gamma$ -chains (50,000 daltons) and light chains (22,000 daltons) in second dimension.

total immunoglobulin synthesis), were also clearly separated from the other heavy chains. Once the position of a chain was established, precipitation with chain-specific sera was not always essential (Fig. 1).

Two-dimensional gel analysis allowed the clear identification of two forms of IgD (precursor, surface) and three different forms of IgM (precursor, secreted, and surface). Although pulse-chase experiments indicate that IgM1 is the precursor of secretory IgM2 (18), it is not yet known whether it is also a precursor of surface IgM3. Surface and secreted IgM may well be products of distinct but closely related genes, or may be due to post-transcriptional splicing leading to two different mRNA species with different carboxy terminal coding sequences (R. Wall and L. Hood, personal communication). Surface and secreted IgM show extensive serologic cross-reaction when xenoantisera are used. They also share some allotypic determinants (22). However, recent data suggest that surface IgM differs from its secretory counterpart by possession of a hydrophobic segment (23). Our demonstration that neuraminidase-treated surface IgM is more acidic than the precursor of secreted IgM is consistent with the idea of a distinct precursor, but by no means proves its existence.

The rate of IgD synthesis in our experiments was very small in relation to IgM or IgA. Incorporation of [ $^{35}$ S]-methionine into IgD was approximately 0.1% of TCA precipitable counts in the cell lysate. For comparison, incorporation of [ $^{35}$ S]-methionine into H-2K or Ia antigens is of the order of 0.75 to 2% of TCA precipitable counts (24, 25). Reliable detection of IgD biosynthesis required considerable attention to detail. In particular, "nonspecific" binding of cellular proteins to staphylococci was found to be highly dependent on pH and ionic strength. Best results were achieved with wash buffers containing 0.05 M Tris, pH 8.3, with 0.4 M NaCl added. The binding of IgG to staphylococcal protein A was equal to that under usual conditions (Goding, unpublished data).

Our results show clear evidence that IgD heavy chains are

synthesized as a cytoplasmic precursor ( $\delta_1$ ) form which is more basic than the surface ( $\delta_2$ ) form. The apparent m.w. are 59,000 and 63,000 to 72,000, respectively. In some experiments (e.g., Figs. 3 and 4), the  $\delta_1$  band consisted of two very closely spaced parallel lines. However, there was no clear-cut evidence for a precursor-product relationship between these forms, since their relative intensities did not change in a predictable way in pulse-chase experiments.

Addition of carbohydrate to glycoproteins is known to occur in several distinct stages. Nascent chains are glycosylated as they form on the luminal side of the rough endoplasmic reticulum (1). The initial carbohydrate chain, which is preformed and donated via a dolichyl diphosphate intermediate, contains *N*-acetyl glucosamine, glucose, and mannose (26). Subsequently, the glucose and some of the mannose residues are removed (21). Shortly before appearance on the surface, galactose and sialic acid residues are added, probably in the Golgi apparatus (27).

We have shown in this paper that the precursor forms of IgM and IgD possess mannose and/or glucose, but lack terminal galactose residues. The surface forms possess terminal galactose, since they bind to *R. communis* hemagglutinin. Neuraminidase treatment showed that the increased acidity of the surface form of IgD could be accounted for by addition of sialic acid. Thus, the glycosylation of IgD occurs in a manner similar to that of the membrane glycoprotein of vesicular stomatitis virus and glycophorin (see Reference 3).

The susceptibility of the precursor form of IgD to endoglycosidase H suggests that it possesses oligosaccharides of the high mannose type. The resistance of the surface form of IgD to cleavage by this enzyme is consistent with its possessing complex type oligosaccharides, and also argues against contaminating protease activity. The apparent m.w. of the endoglycosidase H treated  $\delta_1$  chains (50,000 daltons) suggests that they are made up of four domains rather than five as in  $\mu$ -chains (28).

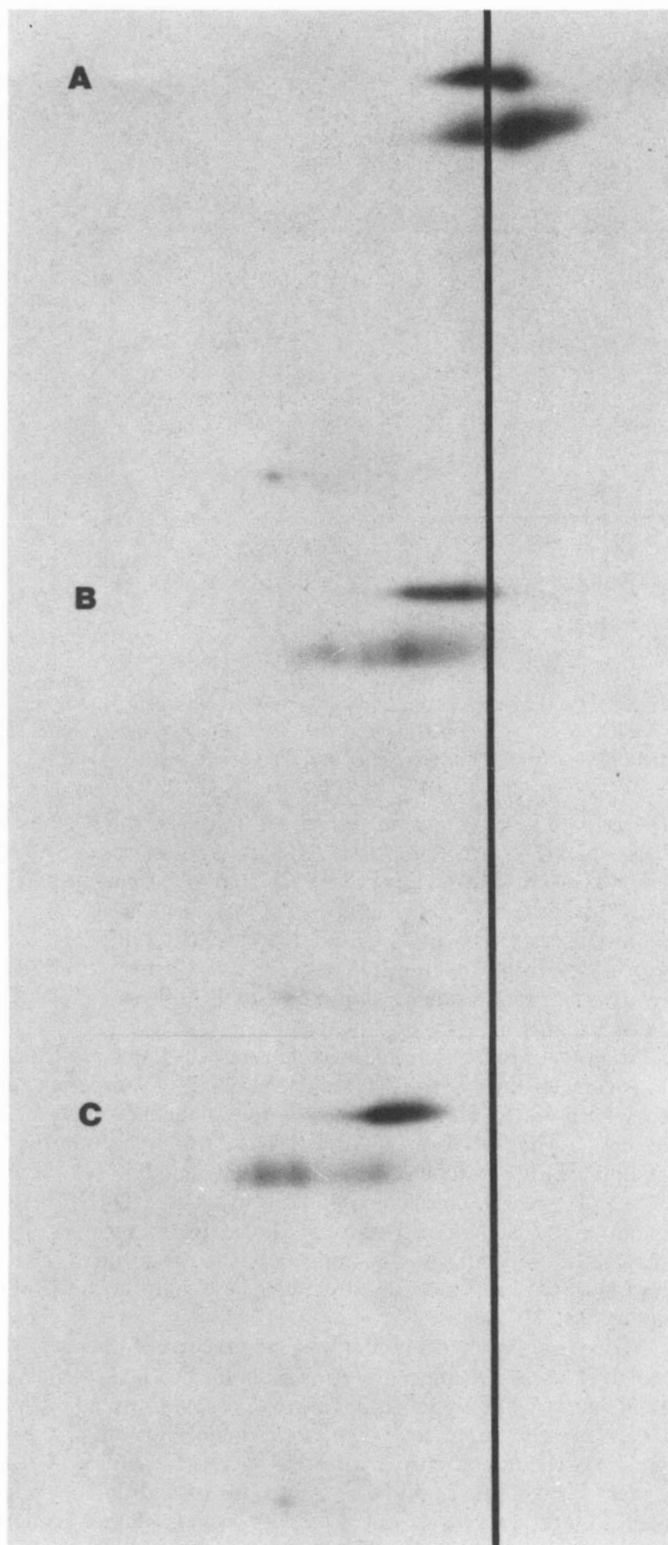


Figure 6. Treatment of lymphocyte surface immunoglobulins with neuraminidase. Spleen cells were labeled with  $^{125}\text{I}$  by the lactoperoxidase technique, lysed in 0.5% Nonidet P40, and immunoglobulins precipitated with rabbit anti-mouse Fab and staphylococci. Pellets were treated with neuraminidase before analysis. A, control (non-neuraminidase); B, 0.01 unit/ml neuraminidase, 1 hr at 20°C; C, 0.1 unit/ml neuraminidase, 1 hr at 20°C. Tenfold increase in concentration of neuraminidase over that used in C produced no further change. Vertical line indicates gel alignment.

Our results suggest that the rates of synthesis of surface IgM and IgD are not greatly different. Ligler *et al.* (28A) found similar rates of recovery of surface IgM and IgD after modulation with anti-immunoglobulin. IgD secretion, if it occurs at all, must be less than 10% of the level of surface IgD biosynthesis, since there was no detectable IgD in the supernatants of biosynthetically labeled cells.

The  $\delta_2$  form seen in two-dimensional gels was heterogeneous in both charge and apparent m.w. This heterogeneity was observed in every experiment, including both those using biosynthetic labeling and lactoperoxidase catalyzed surface radioiodination. The observed heterogeneity probably reflects small differences in neutral sugar and sialic acid composition, which are common in glycoproteins. Many cell surface glycoproteins show even more marked heterogeneity. For example, the T cell alloantigen Thy-1 shows a series of four to five parallel oblique but nearly vertical streaks, which are converted to one oblique, nearly vertical streak after neuraminidase treatment (29). The observed heterogeneity could also be due to partial proteolysis (see Reference 19). However, heterogeneity was always much more marked in the  $\delta_2$  form than in the  $\delta_1$  form. This would imply that the surface form is more susceptible to proteolysis than the cytoplasmic form, but controlled proteolytic digestion showed that the most slowly migrating population of surface IgD heavy chains (presumably the most highly glycosylated) is also the most resistant to proteolysis (30).

The factors that determine whether a protein is secreted or remains in the membrane are still poorly understood (31). Although our data are limited, they do not reveal any obvious differences in carbohydrate structure between the secreted and surface forms of immunoglobulin heavy chains. Recently, mannose-specific receptors have been postulated to be of importance in the clearance of IgM containing immune complexes by the reticulo-endothelial system (32). Similarly, the presence of mannose 6-phosphate on lysosomal enzymes may direct their movement from the endoplasmic reticulum into lysosomes (31). These results suggest a role for carbohydrate in the movement and localization of glycoproteins. It is of interest that the Fc portion of surface IgM and IgD have been shown to bind nonionic detergent (suggesting the presence of a sequence of hydrophobic amino acids) whereas the Fc of secretory IgM does not bind detergent (23).

The major histocompatibility antigens and Ia antigens are known to span the lymphocyte membrane (33), but it is not yet known whether IgD or IgM are transmembrane proteins. We have been unable to detect any evidence of phosphorylation of IgD by using the methods of Pober *et al.* (34; unpublished experiments of J. W. Goding and H. Shulman). If it is accepted that cell surface receptors transmit signals across the membrane, it follows that they may be associated with additional proteins, perhaps inside the cell. One such protein may be the I<sub>i</sub> protein (35), which is precipitated together with murine Ia antigens. In our studies, we found no evidence for a specific association of IgD with any other protein, with the possible exception of actin. Actin was, however, also present in control precipitates, and its significance is unclear (see Reference 36).

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