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IMMUNOGLOBULIN D IN SERUM, BODY FLUIDS AND LYMPHOID TISSUES

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SUMMARY

Measurements of serum IgD concentrations at various ages and in a number of diseases are reported. IgD was not detected in plasma obtained during the first 6 months of life and was only very rarely found in cord plasma. Older children and adults showed a wide range of levels. IgD levels were not increased in three examples of 'autoimmune' disease, but increases were noted in some patients having chronic infections. IgD was usually absent in hypogammaglobulinaemia. IgD was not detected in a variety of body fluids.

Lymphoid cells containing IgD were rare in lymph nodes, spleen and intestinal mucosa compared with cells containing other classes of immunoglobulins. In adenoid tissue IgD containing cells occurred more frequently and were often similar in numbers to those containing IgM.

INTRODUCTION

Immunoglobulin D constitutes a fourth class of human immunoglobulins which is distinct from IgG, IgA and IgM, but has certain structural and other similarities to these proteins (Rowe & Fahey, 1965a). This paper reports IgD levels in serum and other body fluids, and its demonstration in lymphoid cells.

MATERIALS AND METHODS

IgD was detected and estimated using specific antisera raised in rabbits. Rabbits were immunized with D-myeloma proteins using techniques previously described (Rowe & Fahey, 1965b), and rendered specific for IgD by absorption with normal human plasma in which IgD could not be detected, or with IgG. Specificity was assessed by Ouchterlony

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analysis and by immunoelectrophoresis against human sera and preparations of IgG, IgA and IgM. There was no evidence of reactivity with proteins other than IgD. An antiserum to the D-myeloma protein of patient SJ (Rowe & Fahey, 1965b) was used for most of this study. A second antiserum to the D-myeloma protein of patient TN was also used. Antisera to other immunoglobulins were prepared by methods similar to those of Fahey & McLaughlin (1963).

Quantitative estimations of IgD and other immunoglobulins were made by the technique of single radial diffusion in antibody in agar plates (Mancini, Carbonara & Heremans, 1965) using modifications previously described (Rowe & Fahey, 1965a). A series of dilutions of a standard was used to calibrate every plate. The standard most frequently used for IgD was serum WT, IgD 0·33 mg/ml (Rowe & Fahey, 1965a). Another standard containing IgD 57% of WT was used on occasion, but all results are expressed as a percentage of WT. The standard for IgG, IgA and IgM was serum from a single donor, which has previously been described (Rowe *et al.*, 1968). Provisional immunoglobulin concentrations of this standard are IgG 12·5 mg/ml, IgA 3·1 mg/ml and IgM 0·65 mg/ml. Most samples of serum or other body fluids were stored at −20°C before analysis after adding a trace of sodium azide. Diameters of precipitin rings were measured after 24 hr at 4°C. The standard errors of five estimations of IgD on the same samples on different plates on different days was found to be approximately 10% of the concentration when concentrations were 20% or greater. The reproducibility decreased at lower concentrations; the limit of detectability lay between 5 and 2·5% of the standard. Undetectable levels were recorded as less than 1% of the standard.

Saliva was collected with care to prevent admixture with bronchial secretions. After centrifugation to remove mucus the supernatant was concentrated ten-fold by negative pressure dialysis through 8/32 in 'Visking' dialysis tubing. Normal colostrum was analysed without prior treatment or concentration. Intestinal secretions were collected from a small polyethylene tube introduced into the jejunum of fasting subjects. One hundred to 300 ml of fluid, contaminated with bile, was collected into a flask immersed in ice-cold water. After centrifugation the supernatant was concentrated 100- to 300-fold by negative pressure dialysis or by lyophilization after dialysis against distilled water. Urine was concentrated between 120- and 400-fold by dialysis against tap water followed by lyophilization. Lacrimal secretions were obtained from the lower conjunctival sac following stimulation by spirits of ammonia or onions and were not concentrated. Samples of bile were obtained and examined without concentration following cholecystectomy. In addition, unconcentrated samples of mature milk and cerebrospinal fluid (from normal donors and patients with multiple sclerosis) were also examined.

The tissue specimens studied for the presence of cells containing IgD included the following: jejunal or rectal histologically normal mucosal biopsies removed from eleven adult subjects; splenic tissue obtained during a splenectomy performed on two young adults for idiopathic thrombocytopenic purpura; adenoids obtained from five children (aged 4–8) during a tonsillectomy and adenoidectomy as commonly performed on children; abdominal lymph nodes were obtained from four adult patients during laparotomy.

For the immunofluorescence studies the γ-globulin fraction of the specific anti-IgD (raised to SJ D-myeloma protein) was prepared by two successive precipitations with 2 M-ammonium sulphate at pH 6·8. This was conjugated with fluorescein isothiocyanate at pH 9, and the excess of free fluorescein removed by passing the conjugated proteins through

a Sephadex G-25 column eluting with 0·01 M-phosphate buffer, pH 8·0. The fluorescent γ-globulin was absorbed with rabbit liver powder and with washed human group A, B and O red blood cells. An aliquot of the fluorescent γ-globulin was subsequently absorbed with isolated D-myeloma protein from patient BM and was used in control experiments.

Immediately after removal, the tissues were quick-frozen by means of solid carbon dioxide on the holder of a cryotostat. The frozen sections (3–5 μ) were dried and fixed for 3 min in absolute methanol. After gentle washing in 0·02 M-phosphate buffer, pH 7·2, the sections were incubated with the fluorescent antisera in a moist chamber, at room temperature, for 1 hr. The excess of fluorescent dye was then washed out by dipping the slides in successive baths of buffer. Finally, the sections were mounted in buffered glycerine and sealed with a colourless nail polish. Serial sections were usually incubated with specific fluorescent antisera directed against each class of human immunoglobulin (IgA, IgM, IgG and IgD). This procedure and the microscopic examination were performed within 24 hr of obtaining the tissue specimens.

For each biopsy, one section was stained with the May–Grünwald–Giemsa method as used in clinical haematology, since this procedure proved useful for the identification of plasma cells in the preparation.

On intestinal tissue sections, the population densities and mean volumes of plasma cells containing each type of immunoglobulin were obtained by planimetric measurements and by applying the mathematical formulae which have been previously described (Crabbé, Carbonara & Heremans, 1965).

RESULTS

The antigenic determinants of myeloma proteins may not be entirely representative of the related class of 'normal' immunoglobulins. This might affect the use of antisera to myeloma proteins as quantitative reagents. Comparison was therefore made of the reactivity of antisera to two different myeloma proteins. Fifty-three sera from healthy individuals and from hospital patients without myeloma were selected for their range of IgD concentrations as shown by one antiserum, and estimates repeated using a second antiserum (Fig. 1). No discrepant values were obtained, and the differences between values were attributed to the known error of the method.

IgD in serum

Serum from 186 samples of cord blood was examined. IgD was not detected in twenty-three samples obtained in Belgium, seventeen samples obtained in the United States or in 142 samples obtained in Britain. IgD was detected at levels less than 5% in four cord sera obtained in Britain. Since haptoglobin was also present in these four samples, it is possible that the presence of IgD was due to contamination by maternal plasma. In the series from the United States maternal serum was also examined; levels ranged from $<1\%$ to 22%.

Serum from forty-one young children was examined to determine the earliest age at which IgD could be detected. Serum from two sources was available; the first comprised fifteen samples obtained on a random basis from a hospital haematology department, the second comprised twenty-six samples referred to this laboratory on account of suspected antibody deficiency. The age of first appearance of IgD was 7 months (Table 1). IgD levels in the sera of older children and of adults were determined in the groups shown in Table 2. Distribution of levels in some of these groups are shown in Fig. 2. Groups 2–6 comprising

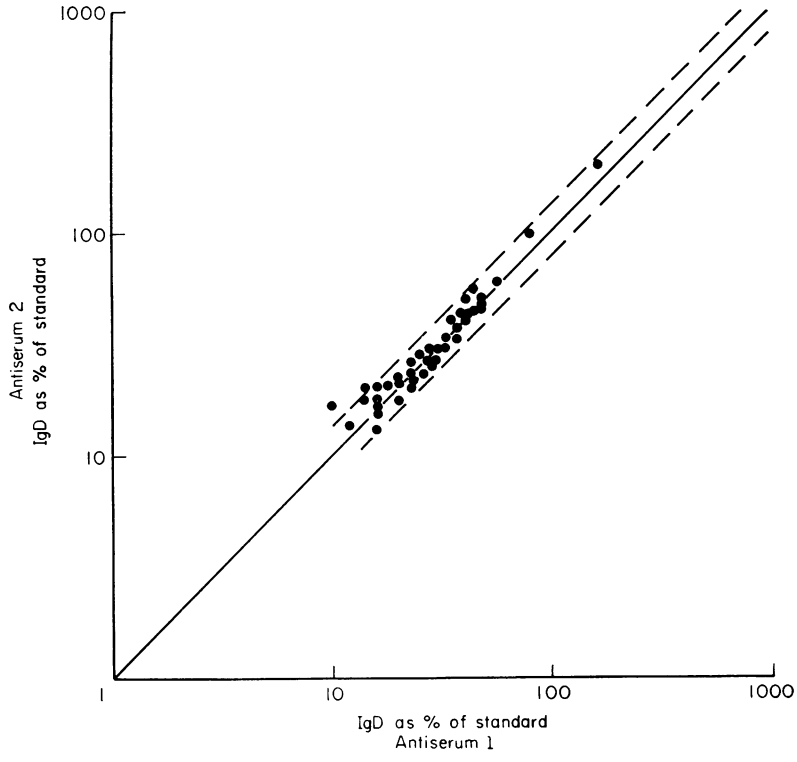

FIG. 1. Comparison of estimations of IgD in fifty-three human sera using antisera to two different D-myeloma proteins. The broken lines indicate ± 1 SD of the quantitative technique.

sera from healthy individuals showed similar distributions. The mean IgD concentration of the 204 individuals of Groups 3 and 5 was 15% corresponding to 0·05 mg/ml. No differences in distributions were observed between the sexes in these groups. Groups 1 and 7 comprised

TABLE 1. IgD in serum from young children

Age (months)	Serum IgD	
	Detected	Undetectable
<1	0	4
1	0	5
2	0	7
3	0	4
4	0	6
5	0	1
6	0	3
7	3	2
8	2	1
9	1	1
10	0	0
11	1	0

unselected sera from hospital patients. These showed similar distributions to the groups of healthy individuals. Group 8 comprising elderly hospital patients showed a rather similar distribution, but there was an increased proportion of individuals in the 1–10% concentration range. These findings indicate that the pattern of distribution of IgD levels is established in childhood, and that no major changes subsequently occur.

TABLE 2. Groups of individuals whose serum IgD was measured

Group	No. in Group	Source	Age (years)
1	61	Random samples from the Birmingham Children's Hospital	8/12 to 15
2	85	Single members of healthy twin pairs (Rowe *et al.*, 1968)	12 to 19
3	95	Medical students and staff of the University of Birmingham	18 to 45
4	42	As Group 2	20 to 70
5	109	Staff and blood donors at the N.I.H., U.S.A.	19 to 57
6	208	Belgian blood donors	Adults
7	400	Random samples from the Queen Elizabeth Hospital, Birmingham	Adults, younger than 60
8	77	As Group 7	Older than 59
9	75	Rheumatoid arthritis	
10	44	Sjögren's syndrome	
11	32	Autoimmune (Hashimoto's) thyroiditis	
12	78	'Idiopathic' hypogammaglobulinaemia IgG < 200 mg/100 ml	
13	145	Sera containing Bence Jones proteins, G- or A- myeloma proteins, or M-macroglobulins of Waldenström	

Values of Group 5 have been reported previously (Rowe & Fahey, 1965a). Sera of Group 12 were pre-treatment samples from patients in the British Medical Research Council's Working Party's Trial of γ-globulin therapy in hypogammaglobulinaemia.

Certain healthy adults had unusually high levels of IgD, e.g. four individuals with levels from 70 to 120% were noted in Group 5, and one British male adult (not included in Group 3), had a level of 77% (his relatives had the following levels; father 16%, mother <1%, brother <1%). It was not possible to identify any factors in their previous history or present state of health which might have been responsible for the high levels of IgD in these individuals.

Immunoglobulin D levels were found to be stable in several healthy individuals over a number of years. For example, levels ranging from 92 to 100% over a 1½-year period and from 7 to 11% over a 3½-year period, were observed in two individuals.

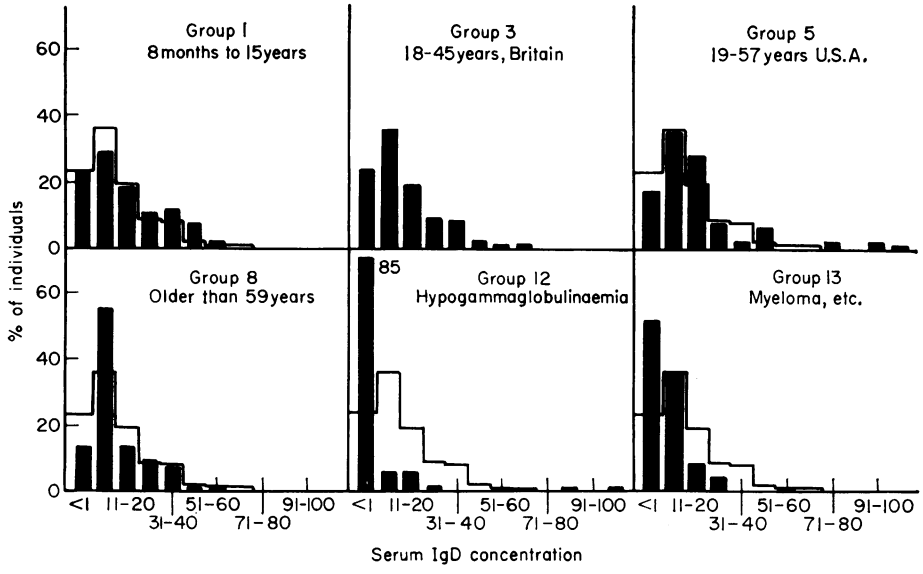

FIG. 2. Distribution of serum IgD concentrations in some groups of Table 2. Concentrations expressed as per cent of standard WT. The continuous line is the distribution of the healthy adults of Group 3.

Serum IgD concentrations were measured in a number of diseases. No systematic increase of IgD levels was seen in rheumatoid arthritis (Group 9), although unusually high levels of other immunoglobulins occurred in some patients (Fig. 3). Similarly no increase of

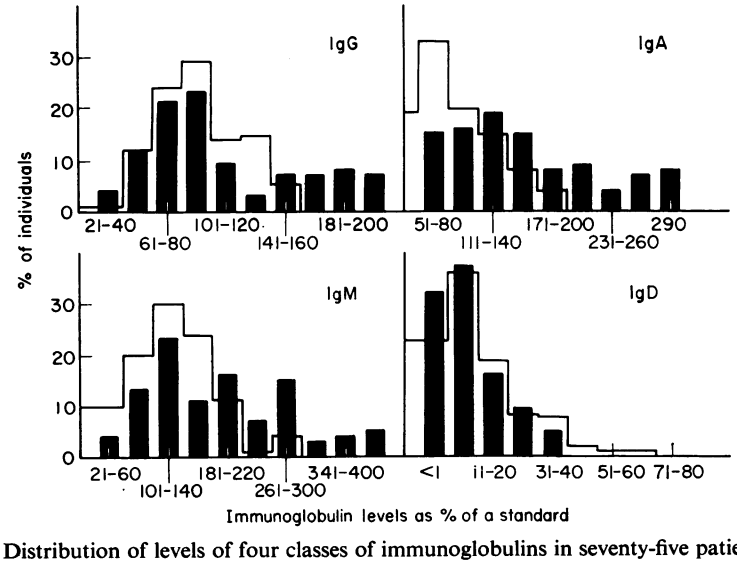

FIG. 3. Distribution of levels of four classes of immunoglobulins in seventy-five patients with rheumatoid arthritis. The distribution in healthy adults (Group 3 of Table 2) is shown as a continuous line. Concentrations of IgD are unchanged in this disease, although concentrations of other immunoglobulins may be increased.

IgD levels was noted in the sera of Groups 10 and 11 from patients with Sjögren's syndrome and autoimmune thyroiditis. Thus in several diseases associated with autoimmune disorders IgD concentrations were unaffected. However, in two disease states where immunoglobulin levels are reduced, IgD was also reduced. In idiopathic hypogammaglobulinaemia (Group 12), the distribution of levels was strikingly altered. The protein was usually undetectable, but occasional high levels were observed. In the plasmacytic disorders of multiple myeloma and Waldenström's macroglobulinaemia (Group 13) levels of 'normal' immunoglobulins are commonly reduced. IgD also showed reduced levels.

IgD in body fluids

Colostrum. Results of immunoglobulin measurements on unconcentrated colostrum and serum from twelve British donors are shown in Table 3. IgD was not detected in any sample.

TABLE 3. Immunoglobulin concentrations in serum and colostrum from twelve donors

Immunoglobulin	Level of immunoglobulin (% of a standard serum)	
	Serum	Colostrum
IgG		
Range	34–100	1.2–25
Mean	63	3.1
IgA		
Range	40–160	84–840
Mean	100	376
IgM		
Range	40–360	5–400
Mean	169	138
IgD		
Range	< 1–39	Not
Mean	9.4	detected

IgA was the predominant immunoglobulin: IgM was also present, and in five of the samples was apparently in higher concentration than in the plasma. Only small amounts of IgG were present. Subfractions of two samples of mature milk which were rich in IgA also lacked IgD.

Saliva. Results of measurements on twenty-eight Belgian hospital patients aged 35–60 years are shown in Table 4. The saliva was concentrated ten-fold before analysis. IgD was not detected, and IgA was predominant. IgD was also not detected in the saliva of eight healthy individuals tested without prior concentration, nor was it found in IgA-rich fractions of saliva prepared by salt precipitation and chromatography on DEAE-cellulose.

Intestinal secretions. Jejunal secretions were collected from twenty-eight hospital patients without gastro-intestinal disease, and were concentrated 100- to 300-fold. Seventeen of the

samples gave no precipitates in antibody in agar plates for IgD. The remainder gave precipitates, but it was not possible to demonstrate their specificity by reactions of identity with IgD in an Ouchterlony system. Such non-specific precipitates may have been the result of interaction between proteolytic enzymes in the sample with the proteins incorporated into the agar. IgD was also not detected in concentrated jejunal secretions from four patients with idiopathic steatorrhoea.

Bile. Eleven samples of bile were obtained at cholecystectomy and were analysed without concentration. Although some of these produced multiple rings in antibody in agar plates it was not possible to demonstrate the presence of IgD by reactions of identity with IgD on Ouchterlony analysis.

TABLE 4. Immunoglobulin concentrations in serum and saliva from twenty-eight donors*

Immunoglobulin	Level of immunoglobulin (% of a standard serum)	
	Serum	Saliva
IgG		
Range	52–160	0–0.74
Mean	86	0.04
IgA		
Range	40–340	0–10
Mean	121	1.92
IgM		
Range	40–220	0–2.0
Mean	100	0.12
IgD		
Range	<1–62	Not
Mean	15	detected

*Measurements made after ten-fold concentration, but expressed here as the level present before concentration.

Urine. Samples from three healthy adult males were concentrated between 120- and 400-fold. One individual had a serum IgD of 57%. IgD was not detected in any of these samples.

Lacrimal secretions. IgD was not demonstrable in concentrated tears from three individuals. One sample, with a high content of lysozyme, formed non-specific precipitates in the antibody in agar plates.

Cerebrospinal fluid. Five unconcentrated samples from individuals without neurological disease and five unconcentrated samples from cases of multiple sclerosis were tested. IgD was not found in any sample.

IgD in lymphoid tissues

IgD was demonstrated in lymphoid cells by the use of fluorescein-labelled specific anti-IgD. The fluorescence was specific for IgD, since the activity of the antiserum was

abolished by absorption with a highly purified D-myeloma protein different from that used to prepare the antiserum. In most tissues, cells containing IgD were rare.

In the lymph nodes and in the spleen, very few cells reacting with fluorescein-labelled anti-IgD were seen. Many fields had to be looked at in order to find a specifically fluorescent cell. Fig. 4 shows such a cell in an abdominal lymph node. One of the lymph nodes examined showed a few weakly but definitely fluorescent germinal centres after incubation with anti-IgD antiserum.

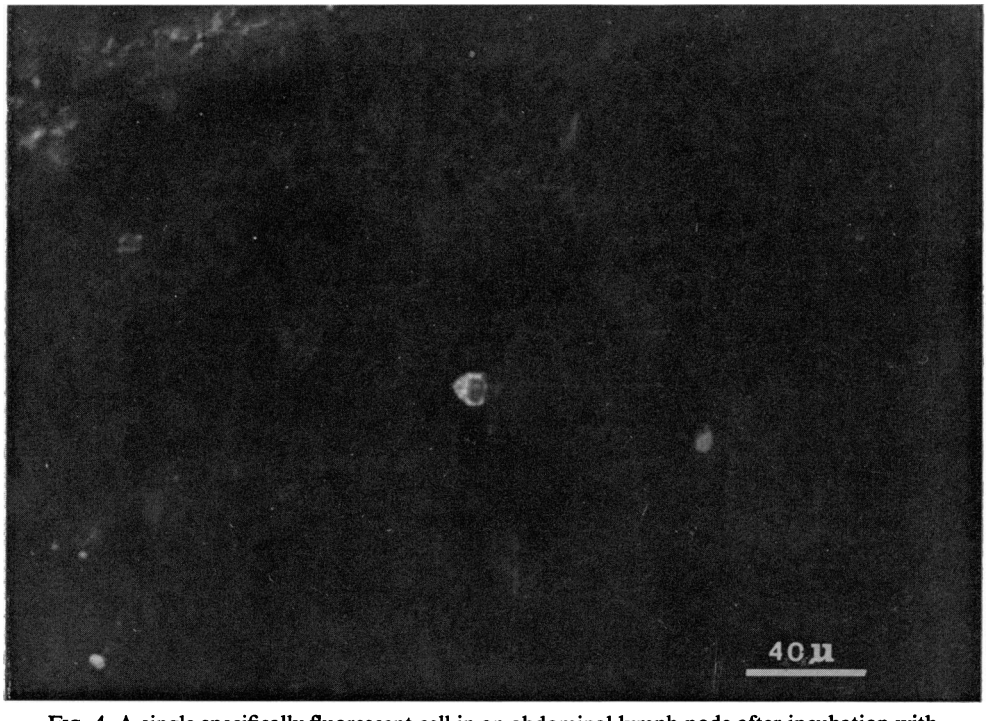

FIG. 4. A single specifically fluorescent cell in an abdominal lymph node after incubation with fluorescein-labelled anti-IgD.

All specimens of intestinal mucosa proved to be very poor in IgD-containing cells (Fig. 5). It was not uncommon for a microscopic section of intestinal mucosal biopsy to be completely devoid of reacting cells. The mean population density of cells containing IgD in the small intestinal mucosa was found to be 2700/mm^3. In Fig. 6 a comparison is given of the population densities of the cells containing all four types of immunoglobulins estimated as described by Crabbé & Heremans (1966). The rectal mucosal tissues studies were also very poor in IgD reacting cells.

The mean volume of the cells containing IgD in the intestinal tract was 620 μ^3. This figure compares with the values previously found for the cells containing IgA, IgM and IgG which were, respectively, 594, 649 and 583 μ^3.

In comparison with the preceding tissues, adenoids were found to be richer in cells containing IgD. This is shown on Fig. 7, while Fig. 8 represents the IgG-containing population in the same area from the next serial section of the same tissue. A better appraisal of the

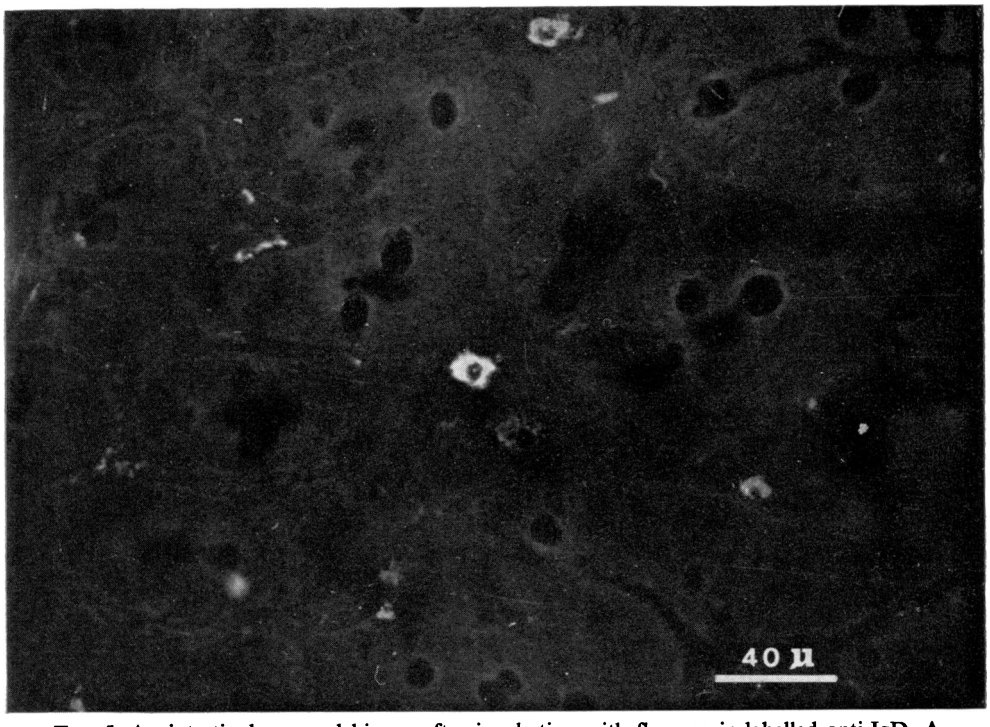

FIG. 5. An intestinal mucosal biopsy after incubation with fluorescein-labelled anti-IgD. A single cell containing IgD lies between several crypts of Lieberkuhn in the centre of the field. The remaining fluorescent cells are auto-fluorescent, and can be recognized by dark field illumination.

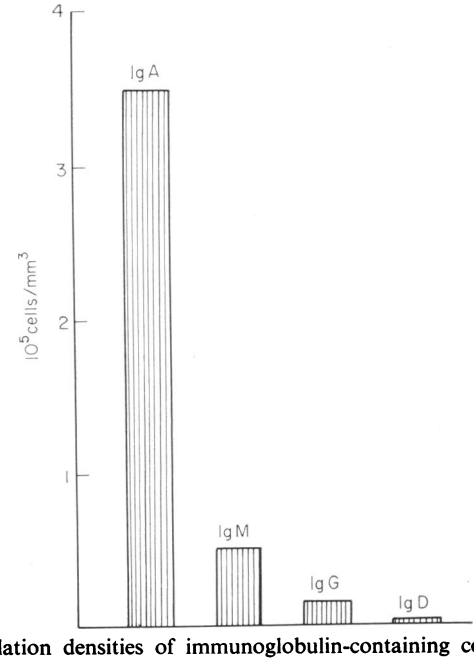

FIG. 6. Mean population densities of immunoglobulin-containing cells in the chorion of intestinal mucosa. IgD containing cells are rare compared with cells containing other classes of immunoglobulins.

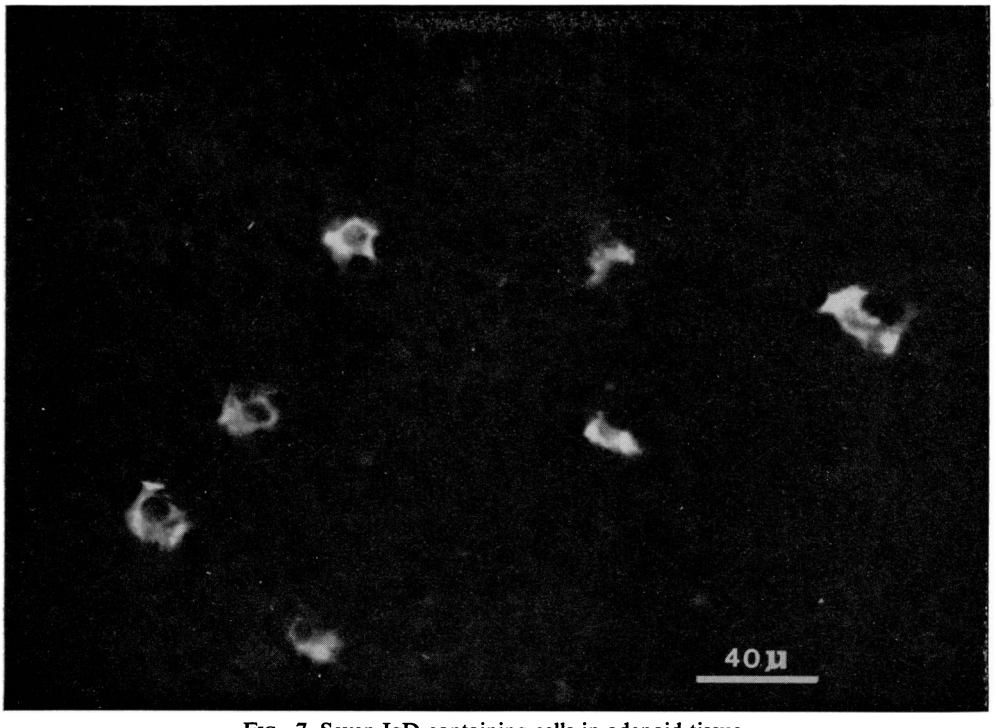

FIG. 7. Seven IgD containing cells in adenoid tissue.

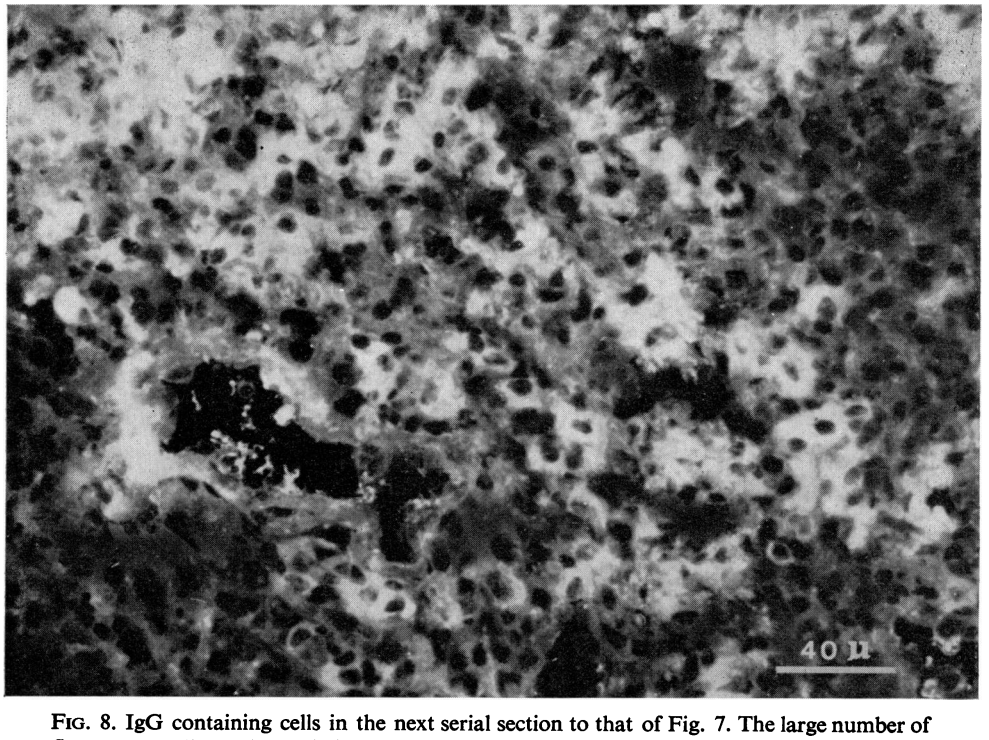

FIG. 8. IgG containing cells in the next serial section to that of Fig. 7. The large number of fluorescent cells render their individual delineation difficult.

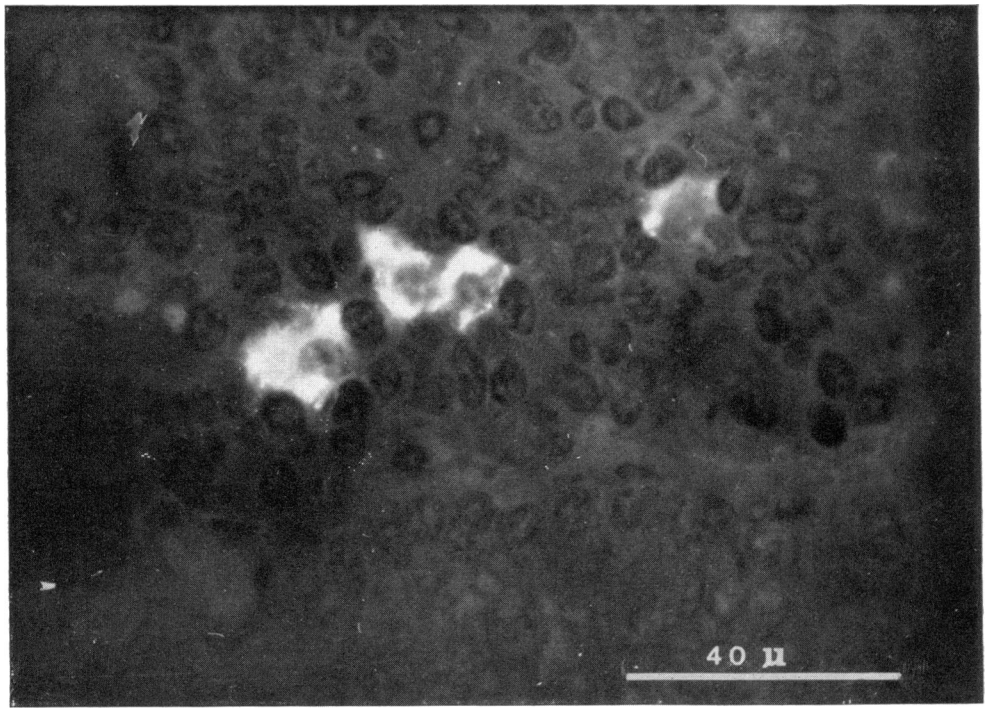

FIG. 9. Higher magnification of IgD-containing cells in adenoid tissue. Note the eccentric nuclei and the relatively large amount of cytoplasm.

morphology of the cells containing IgD can be obtained from Fig. 9, which shows the eccentric location of the nucleus in some cells and the relatively large amount of fluorescent cytoplasm.

The control section, obtained after incubation with fluorescent antiserum which had previously been absorbed with an isolated IgD did not contain any fluorescent cells, except the autofluorescent granular cells. The latter, which corresponded to eosinophils and tissue basophils, were numerous in some samples of intestinal tissue, but could easily be recognized using dark-field illumination.

DISCUSSION

Since the recognition of IgD (Rowe & Fahey, 1965b) further evidence has accumulated that this protein is indeed an immunoglobulin. More D-myeloma proteins have been recognized in patients with typical disease (Burtin, Guilbert & Buffe, 1966; Hobbs *et al.*, 1966; Wiedermann *et al.*, 1967; Fahey *et al.*, 1968). It is remarkable that 80% of these proteins have light chains of antigenic Type L (Fahey *et al.*, 1968). IgD has been demonstrated in occasional cells in the spleens of patients without myeloma (Pernis, Chiappino & Rowe, 1966; Burtin *et al.*, 1966). We here report that the morphology, size and location of these cells is similar to those plasma cells which synthesize other immunoglobulin classes. In IgG deficiency states (hypogammaglobulinaemia) deficiency of other classes of immunoglobulin is common, but in general other plasma proteins are not affected. This study has

shown that IgD is also usually depleted in this condition; however, occasionally elevated levels are found, and in this respect IgD resembles IgM (Barth *et al.*, 1965).

Antibody activity of IgD still remains to be proven. Attempts have been made to demonstrate binding by IgD of diphtheria and tetanus toxoids using radio-gel diffusion analysis (Yagi, Maier & Pressman, 1962). No specific binding by IgD precipitin lines could be demonstrated using plasma from immunized individuals (D. S. Rowe, unpublished observations). The failure to detect binding might be due to only small amounts of antibody activity, or there may be some restriction of time of appearance of IgD antibodies following immunization, or of the type of antigen to which IgD can bind. Although the physico-chemical properties of IgD are similar to those of reaginic antibodies, they are not identical (Ishizaka, Ishizaka & Lee, 1966).

In this study IgD was not detectable in cord serum, except in rare samples where haptoglobin was also found. Thus IgD does not readily cross the placenta, nor is it synthesized by the foetus.

The earliest age at which IgD was detected in children in Britain was 7 months. Comparisons between levels in groups of children and of adults showed no major differences of distribution, and the wide range of levels was similar to that previously reported (Rowe & Fahey, 1965a). This range of concentration is chiefly attributed to differences in rates of synthesis (Rogentine *et al.*, 1966). Although increased concentrations of IgD were found to be associated with certain diseases, it was not possible to relate past infections in healthy individuals to present IgD levels.

Increases in concentrations of other classes of immunoglobulins were not found to be invariably associated with increases of IgD. In this study increased levels of IgD were not found in patients with rheumatoid arthritis, Sjögren's syndrome or Hashimoto's thyroiditis, nor has any change of IgD concentrations been found in experimental malaria infections, despite increases of other immunoglobulins (Tobie *et al.*, 1966). There is, however, suggestive evidence of increased levels of IgD in chronic infections. A high level (100%) was found in a child with chronic osteomyelitis, and levels in five cases of leprosy ranged from 23 to 110%. A level of 200% was found in a child aged 7 with recurrent infections, especially of the skin (Rádl, Masopust & Lacková, 1968). High levels are also found in some children with kwashiorkor (Rowe, Brown & Waterlow, to be published). In a primitive African community IgD was first detectable at the age of 3 months; a wide range of values with frequent high levels was present in childhood, but adults showed the European and North American pattern of distribution (Rowe *et al.*, 1968). IgD was not detected in concentrated urine, even when present in relatively large amounts in the plasma. It was not possible to detect D-myeloma protein in the urine of a patient having protein of this class in the plasma (Harrison *et al.*, 1966). Thus IgD does not readily traverse the normal kidney, nor is it synthesized in the urinary tract.

There is little evidence to suggest that IgD is especially associated with a particular organ or tissue. IgD was not identified in any of the body fluids studied, and the distribution of injected labelled IgD has been found to be 80% intravascular (Rogentine *et al.*, 1966). Plasma cells containing IgD were found in lymph nodes, spleen, intestinal mucosa and adenoid tissue. In the first three locations IgD containing cells were very few compared to the cells containing other classes of immunoglobulins. The larger number of cells containing IgD in the adenoid tissues was probably due in part to the very high density of plasma cells in some areas of the sections, but it was noteworthy that the population density of

IgD containing cells appeared similar to that of IgM. An unusual rectal biopsy showing large numbers of IgD containing cells in a case of ulcerative colitis has also been reported (Crabbé & Heremans, 1966b). The significance of increased IgD synthesis at these sites is at present unexplained; it may be related to localized chronic infection.

The findings reported and reviewed here provide further evidence that IgD is an immunoglobulin. They suggest that IgD synthesis may be stimulated by chronic infection, especially in children. The specific role of IgD in the immune response remains to be elucidated.

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