A NEW CLASS OF HUMAN IMMUNOGLOBULINS

II. Normal Serum IgD

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Investigations of the anomalous proteins associated with multiple myeloma and related diseases have facilitated recognition of separate classes of immunoglobulins. Waldenström's studies in macroglobulinemia (1) emphasized the existence of IgM (γ_{1M}-, β_{2M}-macroglobulins). A distinctive class of myeloma protein of beta globulin electrophoretic mobility and characteristic antigenic properties was recognized (2–6) prior to the identification of the corresponding IgA (γ_{1A}-, β_{2A}-globulins) by immunoelectrophoresis of normal serum (7). Bence Jones proteins, pathological counterparts of γ-microglobulins, were recognized for over 100 years before the corresponding normal proteins were identified (8).

In the course of studies of human myeloma proteins, a unique myeloma protein was identified in the serum of a patient S. J. (9). This S. J. myeloma protein was distinguished from typical G (γ_2) myeloma proteins, A (γ_{1A}) myeloma proteins, M macroglobulins of Waldenström, and Bence Jones proteins in several parameters (9). In the light of the historical evidence that anomalous proteins are closely related to normal serum components, a thorough study of normal proteins was undertaken in an effort to find a normal counterpart to the unique S. J. myeloma protein. This effort was successful and this paper describes a previously unrecognized class of immunoglobulins.

Proteins of this new class are here referred to as IgD, on account of many features which indicate that they are members of the immunoglobulin family, as well as other properties indicating that they constitute a separate and previously unrecognized class of immunoglobulin.

Materials and Methods

Ouchterlony analysis and immunoelectrophoresis were performed by standard methods (10). Antiserums to IgD were prepared by immunizing rabbits with S. J. myeloma protein as described in the accompanying paper (9). The antiserums were made specific for IgD by

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absorption in three different ways: First by absorption with a G myeloma protein of type L, followed by absorption with agammaglobulinemia serum; secondly, by absorption with IgG from normal serum followed by absorption with agammaglobulinemia serum; and thirdly by absorption with a small volume of normal human serum. Similar results were obtained using all three absorbed antiserums. The antiserum used for most of these studies was prepared by absorption of 5 ml of antiserum to S. J. myeloma protein with 0.5 ml of a normal human serum of low IgD level. Antiserums to IgG, IgA, and IgM, and to type K and type L Bence Jones proteins have been described (10).

Gel filtration on sephadex G-200 was performed using a 4 x 46 cm column equilibrated in 0.2 M NaCl, 0.2 M pH 8.0 tris HCl. Material to be fractionated was applied to the column in 20 per cent sucrose to ensure an initial narrow band. The proteins were eluted with the same buffer, and collected as 6 or 10 ml fractions.

For chromatography on diethylaminoethyl (DEAE) cellulose, 5 ml of serum was dialyzed against 0.015 M pH 8.0 potassium phosphate buffer, and applied to a 2 x 22 cm column containing 8 gm of DEAE cellulose equilibrated with the same buffer. Elution was performed using 500 ml of buffer in a gradient from 0.015 to 0.3 M at pH 8.0 (10). For the isolation of IgD 12 ml of serum from patient C. C. was fractionated in the same manner.

Quantitative Determination of IgD.—Levels of IgD and other immunoglobulins were determined by diffusion tests in agar plates with specific antiserum incorporated into the agar (11, 12). The procedure employed has been described in detail (12). Specifically for IgD determination serum or serum fractions were added to small holes in the antibody-agar plate and allowed to diffuse for 16 hours at 6°C. The diameter of the circular precipitin rings which developed around the holes was measured. Values for protein concentration were obtained by comparison of these ring diameters with the diameters of rings produced by a series of concentrations of the protein in a standard reference serum in other holes on the same plate. The specific antiserum for IgD estimations was diluted 40-fold in the antibody-agar plate. A buffer of 0.3 M pH 8.0 potassium phosphate was used in preparing the plates to avoid the formation of non-specific precipitates around the holes which sometimes appeared when buffers of lower salt content were used.

A normal serum of relatively high IgD content was selected for use as a standard. A series of dilutions of this serum was prepared which yielded precipitin rings in the agar plate ranging from 6.0 mm for undiluted serum to 3.3 mm for the serum diluted to 1:40. Dilutions of this serum between 1:50 and 1:100 gave detectable precipitates which were not, however, measurable as rings. Reactions of this intensity were recorded as 1 per cent of the concentration of the standard serum. The IgD content of the standard serum was determined by comparison with purified preparations of the S. J. myeloma protein and the isolated IgD from serum of patient C. C., prepared as described below. Estimates of the concentration of these proteins were based on optical density measurements at 280 mμ, assuming an extinction coefficient of 13.5. The IgD concentration in the standard serum was found to be 0.34 mg/ml when compared with S. J. myeloma protein, and 0.32 mg/ml when compared with isolated IgD. Although some error might be anticipated in the comparison with the S. J. myeloma protein because of myeloma specific determinants reactive with the antiserum, this error was not large, since the values obtained with the two protein preparations were similar. The mean value of 0.33 mg/ml was assigned to the standard serum. Errors may arise in this quantitative method if the proteins being measured differ in molecular size (12). This problem does not arise, however, in the case of IgD since these proteins appear to be homogeneous in size as indicated by a single peak on sephadex G-200 filtration of three normal serums (see Results).

RESULTS

Identification of a Normal Serum Globulin (IgD) Antigenically Related to the S. J. Myeloma Protein.—Immunochemical techniques were used to demon-

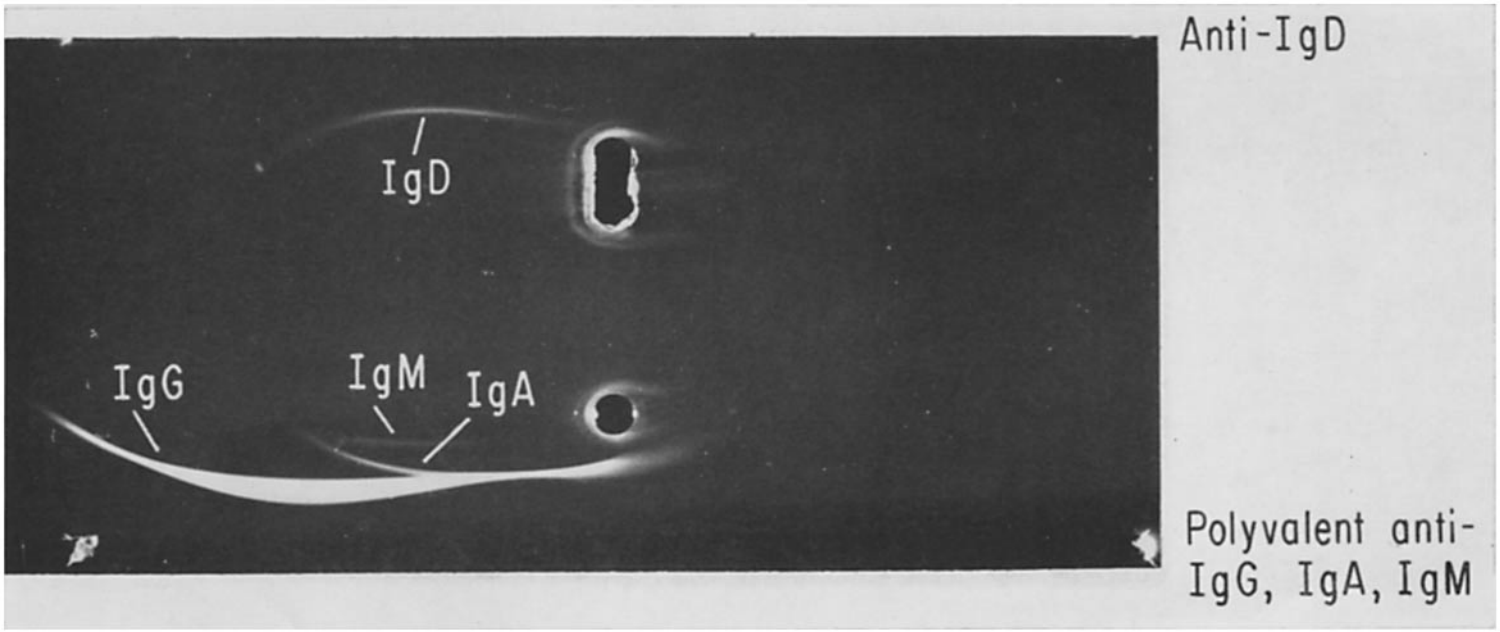

FIG. 1. Demonstration of IgD in normal human serum by immunoelectrophoretic analysis. Specific antiserum reveals IgD as a precipitin line in the fast γ-region.

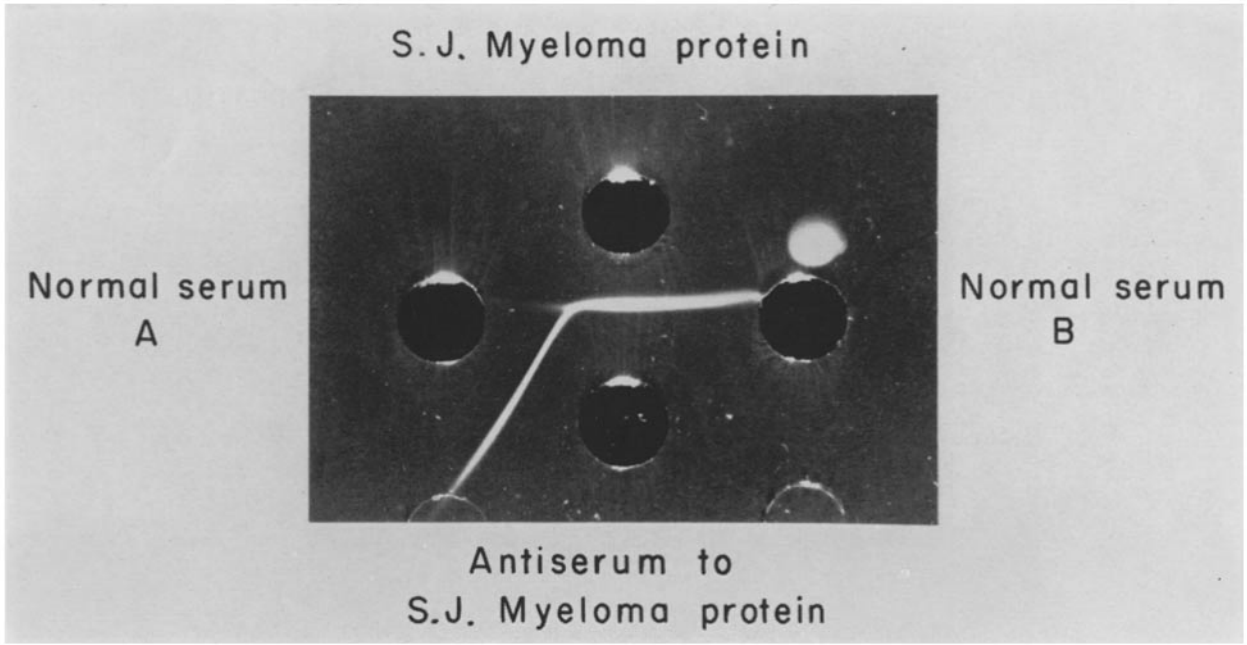

FIG. 2. Antigenic relationship of IgD from normal serum to S. J. myeloma protein. IgD in normal serum A reacts with the specific antiserum, forming a precipitin line which joins the S. J. myeloma precipitin line, indicating common antigenic determinants. The line due to S. J. myeloma protein spurs over that of normal IgD, indicating additional antigenic determinants on the myeloma protein. No IgD is detected in normal serum B.

strate a protein in normal serum which is antigenically related to the S. J. myeloma protein. The immunochemical technics required antiserum reacting only with the new class of immunoglobulin. Such specific antiserums were prepared by immunizing rabbits with the purified S. J. myeloma protein and by absorbing the antiserum so that they did not react with IgG, IgA, or IgM or the non-immune globulins of serum (see Materials and Methods).

Immunoelectrophoretic analysis of appropriate normal serums, developed with specific antiserum to S. J. myeloma protein, revealed a single precipitin

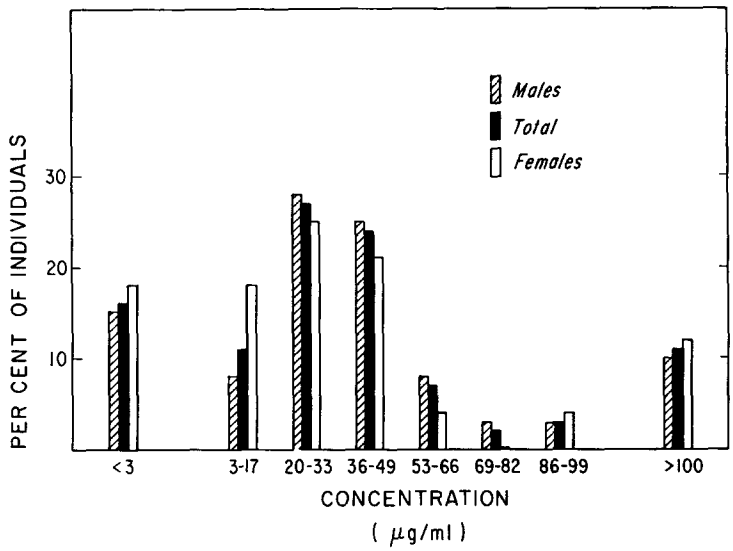

FIG. 3. Distribution of IgD concentrations in serums from 72 normal adult males and 28 normal adult females.

arc in the normal serum (Fig. 1). Ouchterlony analysis in Fig. 2 shows that normal serum A gave a single precipitin line with the specific antiserum. The precipitin line formed in normal serum joined the precipitin line given by the isolated S. J. myeloma protein and indicated that the two proteins shared specific antigenic determinants; *i.e.*, the normal serum globulin was antigenically related to the S. J. myeloma protein.

Concentration of IgD in Normal Human Serums.—IgD concentrations were measured by a quantitative gel diffusion method, using antiserum specific to the protein. Serums were obtained from 72 normal males, aged 19 to 57 years, and 28 normal females, aged 20 to 54 years. The serum IgD concentrations of this group varied widely (Fig. 3), ranging from less than the limit of detectability, *i.e.*, <0.003 mg/ml, to as high as 0.4 mg/ml. The values were not distributed in a Gaussian manner within this range (Fig. 3). Sixty-nine per

cent of the serums had values between 0.02 and 0.05 mg/ml. The median serum IgD concentration was 0.03 mg/ml.

IgD concentrations showed similar distributions in both male and female populations (Fig. 3). There was no correlation between age and concentration (rank correlation = 0.09, $p > 0.1$). A group of 17 healthy children between the ages of 2 and 11 years were also studied and found to have values similar to

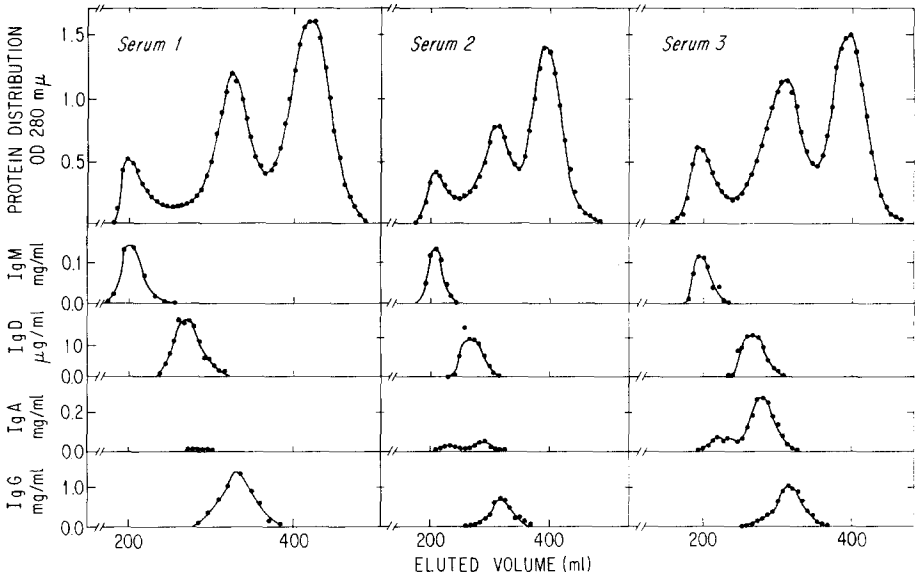

FIG. 4. Distribution of immunoglobulins of three normal serums following gel filtration on sephadex G-200. Serum 1 contained IgG 12.5 mg/ml, IgA 0.14 mg/ml, IgM 1.5 mg/ml, and IgD 0.33 mg/ml. Serum 2 contained IgG 6.1 mg/ml, IgA 0.65 mg/ml, IgM 1.05 mg/ml, and IgD 0.15 mg/ml. Serum 3 contained IgG 10.6 mg/ml, IgA 6.8 mg/ml, IgM 1.4 mg/ml, and IgD 0.16 mg/ml. After determination of optical density the fractions eluted from the column were uniformly concentrated 8 times by ultrafiltration. Immunoglobulin concentrations were estimated by the antibody-agar plate technic. Results are shown as the concentration calculated to be in each fraction prior to ultrafiltration.

the adult levels. Levels ranged between 0.007 and 0.17 mg/ml, the median was 0.03 mg/ml. There was no significant correlation of serum level with the age of these children (rank correlation = 0.34, $p > 0.1$). IgD was not detected, however, in the serums of two healthy infants aged 4 months.

Physicochemical Characteristics.—Immunoelectrophoresis (Fig. 1) revealed normal IgD in the fast γ-region. The form of the IgD precipitin line differs from that of the other three immunoglobulins (Fig. 1). IgD are electrophoretically heterogeneous.

Gel filtration on sephadex G-200 columns was used to characterize IgD and

the other normal serum immunoglobulins (Fig. 4). Three normal serums were fractionated with similar results. The serum proteins were eluted in three major peaks corresponding to the 18S, 7S, and 4.5S components of normal serum. IgD was eluted from the column as a fairly symmetrical peak in fractions midway between the first and second optical density peaks (Fig. 4); *i.e.*, in a

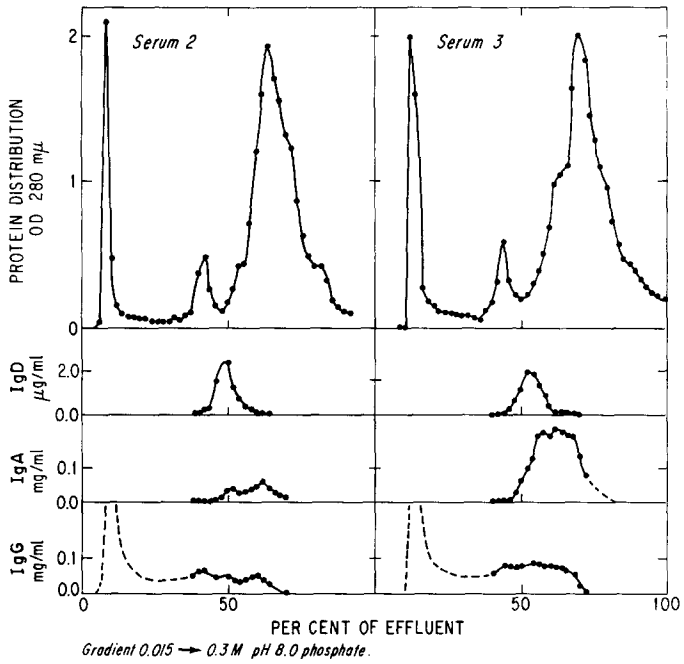

FIG. 5. Distribution of immunoglobulins of two normal serums following chromatography on DEAE cellulose columns (serums 2 and 3 of Fig. 4). After determination of optical density, Ouchterlony analysis was used to localize IgD in the fractions. Fractions from the region in which the protein was detected were uniformly concentrated 10-fold by ultrafiltration, and their concentration of immunoglobulins then determined. Results are shown as the concentration calculated to be in the fraction prior to ultrafiltration. IgM was not detected in the concentrated fractions representing 35 to 70 per cent of the effluent but was localized in fractions eluted later from the column.

region between (and separate from) IgM and IgG. IgD was also distinct from IgA in their manner of elution from the columns. IgD was eluted as a single peak, with no evidence of heterogeneity of molecular size, whereas IgA was eluted over a broader range of fractions and showed two concentration peaks (Fig. 4, serums 2 and 3).

Chromatography on diethylaminoethyl (DEAE) cellulose columns was also used to characterize IgD. Two normal serums were fractionated with similar results (Fig. 5). IgD were eluted as a symmetrical peak in a region

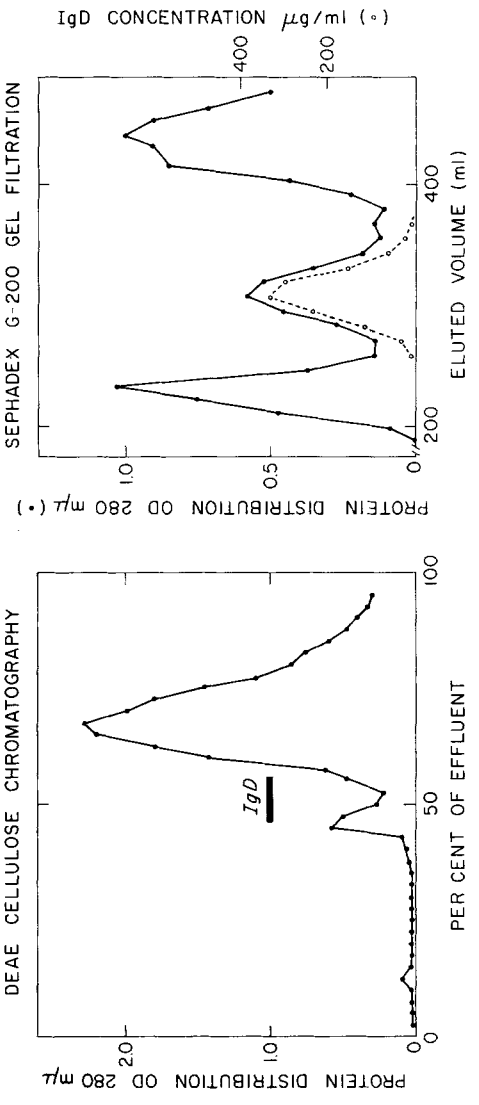

FIG. 6. Isolation of IgD from serum C.C. deficient in IgG and IgA. IgD was eluted in the indicated fractions following DEAE chromatography. These fractions were pooled, concentrated 15-fold by ultrafiltration, and applied to a sephadex G-200 column. The first optical density peak contained IgM, the second peak IgD, and the third peak contained transferrin. Concentrations of IgD in the sephadex eluates were measured by the antibody-agar plate technic.

intermediate between the second optical density peak, which contains transferrin, and the third optical density peak, which includes the bulk of the serum albumin. IgD was clearly different in chromatographic properties from other classes of immunoglobulin. Most of the IgG was eluted in the first chromatographic peak. IgM is eluted after IgD. The IgD and IgA are eluted close together and with some overlapping. IgD, however, appeared in earlier eluate fractions and formed a narrower peak than did IgA (Fig. 5); differences which are consistent with the narrower range and slower mobility of IgD compared with IgA on immunoelectrophoretic analysis (Fig. 1).

Preparation of IgD.—IgD was prepared from normal serum by the technics noted above but in each case the preparations contained IgA and IgG. Although the absolute amounts of these contaminants were low, they constituted a significant proportion of the preparations because only small amounts of IgD were present in the normal serum from which preparations were made. To avoid the difficulties of separation from IgG and IgA, IgD has been isolated from the serum of a 15-year-old patient C. C., who was deficient in IgG and IgA. The IgD and the IgM concentrations in this serum were relatively high. Details of this patient's illness are reported elsewhere (13).

Twelve ml of serum were fractionated by DEAE cellulose chromatography (Fig. 6). The first (IgG) peak was very much reduced. IgD was eluted in fractions in the same region as Fig. 4, and IgG and IgA could not be detected in these fractions. The IgD fractions were pooled and concentrated to 3 ml, and then applied to a sephadex G-200 column. Three protein peaks appeared in the eluate of this column (Fig. 6), and IgD was confined to the second peak. Fractions from this peak were pooled, and concentrated by ultrafiltration to an optical density of 1.60 at 280 mμ.

Ouchterlony analyses (Fig. 7) indicated that the isolated protein contained specific IgD antigenic determinants and was not contaminated by IgG, IgA, and IgM. A horse anti-human serum indicated an absence of non-immunoglobulin contaminants.

Distinctive Antigenic Properties.—The antigenic distinctiveness of IgD was shown by several tests. Antiserum reacting specifically with IgD failed to react with normal IgG, IgA, or IgM (Fig. 7). Similarly, antiserums specific for each of these proteins failed to react with isolated IgD (Fig. 7).

The antigenic relationship of normal IgD and the S. J. myeloma protein was typical of a myeloma protein and the corresponding normal immunoglobulin. Ouchterlony analysis (Fig. 2) showed that the S. J. myeloma protein formed a precipitin line which extended as a spur beyond the precipitin line formed by IgD of normal serum. This spur formation is typical of Ouchterlony test results when a myeloma protein is compared with normal immunoglobulin using antiserum prepared against the myeloma protein (14). The spur indicates that the myeloma protein has one or more antigenic determinants that are

either not present in normal immunoglobulins or are present in very low concentration (14).

Antigenic Determinants of L Polypeptide Chains in IgD.—Isolated IgD from C.C. serum was tested with two antiserums specific to type K (I) light chain

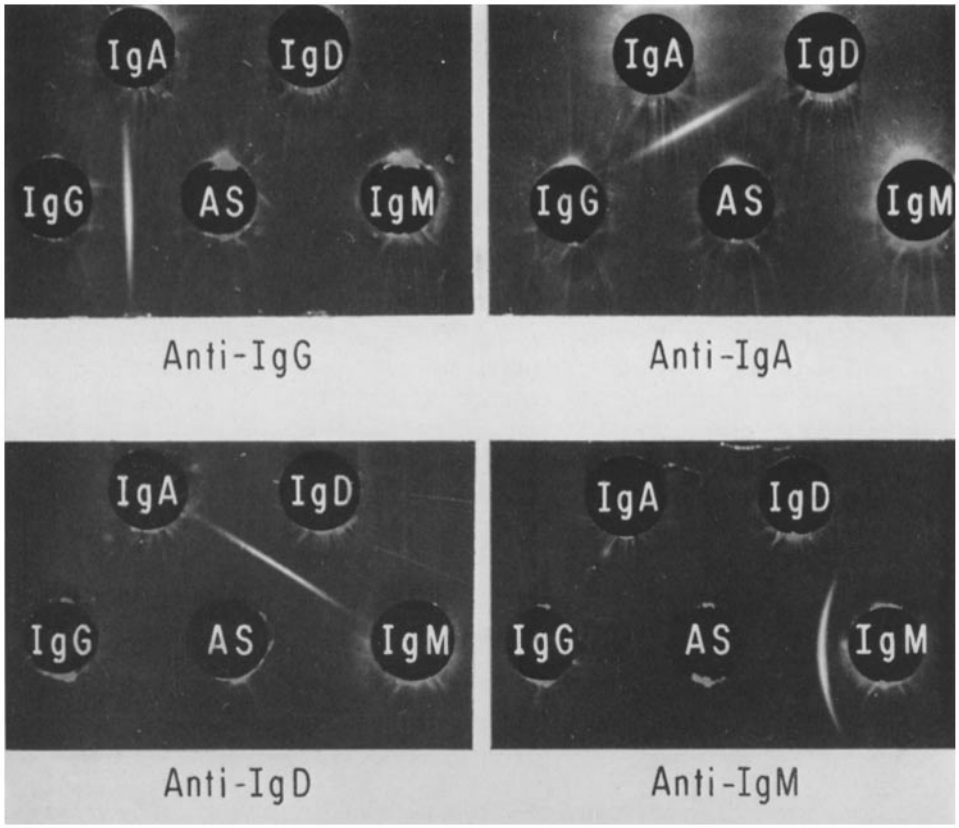

FIG. 7. Ouchterlony analysis showing antigenic distinctiveness of isolated IgD. IgG, IgA, and IgM were obtained from normal serum, IgD from C.C. serum. Protein concentrations were 0.2 mg/ml. The antiserum (*AS*) is designated below each test. Antiserums specific for IgG, IgA, and IgM fail to react with isolated IgD, but do react with proteins of appropriate class isolated from normal serum. Specific antiserum to IgD reacts with the isolated protein, but not with the other immunoglobulins.

determinants and two antiserums specific to type L (II) light chain determinants. Under appropriate conditions, all four antiserums reacted with IgD or its constituent polypeptide chains (Table I). This indicates that IgD possesses type K (I) and type L (II) light polypeptide chains. In this characteristic, IgD is similar to normal IgG, IgA, and IgM.

A series of observations, summarized in Table I, indicated that some of the type K (I) and type L (II) antigenic determinants in the intact molecule were not able to yield precipitin lines with specific antiserum. One of the antiserums to type K (I) determinants precipitated isolated IgD; the second did not. This latter antiserum also failed to react with reduced and alkylated IgD, but did precipitate with light polypeptide chains after dissociation of the constituent polypeptide chains by acidification with 1 M acetic acid. Similar results were

TABLE I

Ouchterlony Analyses of Isolated IgD and S. J. Myeloma Protein and Their Separated Polypeptide Chains

	IgD				S. J. myeloma protein			
	Anti-type K (I)		Anti-type L (II)		Anti-type K (I)		Anti-type L (II)	
	Anti-BJ	Anti-IgG (abs)	Anti-BJ	Anti-IgG (abs)	Anti-BJ	Anti-IgG (abs)	Anti-BJ	Anti-IgG (abs)
Isolated protein	0	+	+	+	0	0	0	0 or +*
Reduced and alkylated	0	+	+	+	0	0	+	0 or +*
Reduced, alkylated, and acidified	+	+	+	+	0	0	+	+
Reduced, alkylated, acidified, and neutralized	+	+	+	+	0	0	+	+

Proteins were tested after reduction and alkylation, and reduction, alkylation, and acidification in 1 M acetic acid according to the method of Fleischman, Pain, and Porter. These preparations were neutralized by dialysis for 16 hours at 4°C against 0.14 M NaCl, 0.1 M pH 8.0 potassium phosphate buffer.

+ indicates a precipitin line, 0 indicates no reaction. Agar plates prepared with 0.3 M pH 8.0 potassium phosphate buffer. Anti-type K (I) antiserums were prepared by immunizing rabbits with type K (I) Bence Jones protein (anti-BJ) or by absorbing antinormal IgG by a Type L (II) G myeloma protein (Anti-IgG abs). Anti-type L (II) antiserums were similarly prepared.

* Some antiserums gave no reaction (0) and others produced a positive reaction (+) with S. J. myeloma protein.

obtained on studying the type L (II) light chain determinants of the S. J. myeloma protein. These were inactive with both antisera to type L (II) determinants prior to reduction and alkylation, and only became strongly reactive after separation of light and heavy polypeptide chains.

These findings show that the light polypeptide chains of IgD possess antigenic determinants in common with type K (I) and type L (II) Bence Jones proteins. Certain of these are apparently unreactive in the intact molecule but become reactive following separation of light from heavy polypeptide chains. Similar observations have been made on other immunoglobulins (15–

18) and indicate that such "hidden" light chain determinants are not unique to IgD.

DISCUSSION

A distinct class of human globulins has been identified and designated IgD. Many features indicate that IgD proteins are members of the immunoglobulin family. Isolated normal IgD contains antigenic determinants of type K (I) and type L (II) light polypeptide chains. Since such chains are constituents of all classes of immunoglobulins, their presence in IgD proteins is strong evidence for regarding these proteins as immunoglobulins. The electrophoretic mobility and the size characteristics of IgD also are similar to other classes of immunoglobulins. IgD was not detected in agammaglobulinemic serums, nor in the serum of young infants. In cases of multiple myeloma and of macroglobulinemia with reduced concentrations of other normal immunoglobulins, IgD was usually undetectable. A myeloma protein, related to normal IgD has been identified (9). Elevated serum levels of IgD have been found in some patients with chronic infectious diseases. These observations, together with evidence from the structural analyses of the protein, indicate a close relationship between IgD and other proteins of the immunoglobulin system.

One of the major distinctive features of IgD is antigenic specificity. The antigenic specificity of IgD is associated with distinctive physicochemical properties. Although IgD shares some properties with other immunoglobulins which migrate in the γ_1-electrophoretic region, studies with sephadex gel filtration and DEAE cellulose chromatography show that each immunoglobulin class is eluted in a different manner. Heterogeneity of IgG and IgA, however, prevents isolation of IgD by the usual methods of serum fractionation.

IgD does not possess the specific antigenic determinants characteristic of IgG, IgA, or IgM and thus does not appear to be a subclass of one of the three known immunoglobulin groups. In addition, IgD can be distinguished from the γ_{2a}-, γ_{2b}-, and γ_{2c}-subclasses of IgG which are recognized by monkey antiserums (19–24). Antiserums specific for these three subclasses of IgG (25) do not react with IgD preparations. Also, antiserum specific for IgD does not react with representative γ_{2a}-, γ_{2b}-, or γ_{2c}-myeloma proteins or with a fourth subclass tentatively designated γ_{2d} (26) or with representative proteins of the four IgG subclasses described by Grey and Kunkel (22). This immunochemical evidence indicates that IgD is a separate class of human immunoglobulin.

The terminology IgD is proposed for the new class of serum protein described here: Ig, on the basis of evidence that these proteins are members of the immunoglobulin family, D because of the evidence that the distinctive properties lie in the heavy polypeptide chains and the properties are sufficiently unique to clearly differentiate these proteins from IgG, IgA, and IgM classes, or from known subclasses of these groups.

Normal serum immunoglobulins can be classified on the basis of the major categories of heavy and light polypeptide chains. Six commonly recognized immunoglobulins are formed by combination of κ (type I) or λ (type II) light chains with γ, α, or μ heavy chains (Fig. 8). The present studies, and related studies of the S. J. myeloma protein (9), indicated that a fourth type of heavy polypeptide chain, δ, may be associated with κ or λ (type I or type II) light chains. With the addition of two types of IgD molecules, the number of normal serum immunoglobulin forms is increased to eight. These are schematically shown in Fig. 8.

Many specific properties of individual classes of immunoglobulins depend on the properties of their heavy polypeptide chains. Thus, the inference that the IgD has distinctive heavy polypeptide chains, δ, raises the possibility that these proteins will have a specific and distinctive role in the immune response.

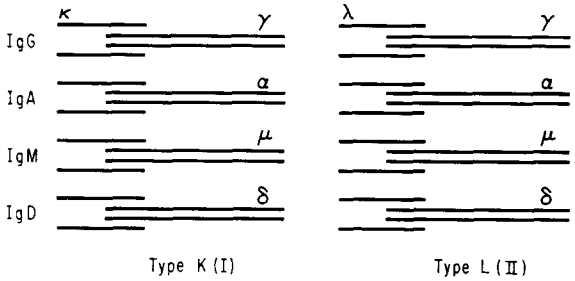

FIG. 8. Schematic representation of the light and heavy polypeptide chains associated with eight forms of normal serum immunoglobulins.

Studies are underway to determine the function of IgD and to compare the biological properties of these proteins with those of other members of the immunoglobulin family.

The wide range of serum levels of IgD found in normal populations remains to be explained. Individual normal serums showed over 100-fold variation in content of this protein, a much wider range from that observed for other classes of immunoglobulin (12). Preliminary studies indicate, however, that the serum level in one individual may remain constant over a period of years. Genetic factors, as well as environmental factors, may help to fix the serum level of IgD but additional studies will be needed to assess properly these possibilities.

SUMMARY

A new class of immunoglobulin, IgD, was identified in normal human serum by immunochemical technics. Antiserums prepared against the unique S.J. myeloma protein facilitated recognition of the related normal protein. IgD was shown to possess type K (I) and type L (II) light chain determinants, similar

to those present in other classes of immunoglobulins. IgD does not possess determinants which are specific to IgG, IgA, or IgM. The IgD proteins possess their own specific antigenic determinants. IgD migrates in the fast γ-region on immunoelectrophoresis. The properties on sephadex gel filtration and DEAE cellulose chromatography are described.

IgD was found to have a median level of 0.03 mg/ml in 100 normal serums. The range of concentrations found in individual normal serums is much wider, however, than that of other classes of immunoglobulins. IgD, on the average, accounts for less than 1 per cent of the normal serum immunoglobulins.

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