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Uneven Distribution of Antitreponema Antibody Activity in Differing Immunoglobulin G Fractions from Patients with Early Syphilis

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Three fractions, containing immunoglobulin G's (IgG's) of differing relative electrophoretic mobility, were isolated from sera of 33 male patients with untreated early syphilis. Serological testing, employing four different serodiagnostic procedures for these fractions at equal amounts of IgG, revealed a very constant reaction pattern for all patients. In the earliest stages of the disease, the most basic fraction was the first to show antibody activity. In progressive stages, antibody activity subsequently was also found in the two less basic fractions. However, in all stages of disease studied, the basic part of the IgG made the largest contribution of the total antibody activity within the IgG class, indicating an uneven distribution of antitreponema antibody activity over heterogeneous IgG. Several possible explanations of the observations are discussed.

Hyperimmunoglobulinemia develops during early syphilis, affecting at least immunoglobulin M (IgM), IgG, and IgA immunoglobulin classes (3; H. E. Menke, Ph.D. thesis, Erasmus University, Rotterdam, The Netherlands, 1975). During infection a variety of antibodies are demonstrable which can react with *Treponema pallidum* or antigens derived from it. However, the presence of these antibodies does not prevent the disease from taking a chronic course in some patients. Investigations of the cell-mediated immune system in early stages of syphilis in humans suggest an impairment of the T-cell branch. This includes a reduced in vitro phytohemagglutinin stimulation of peripheral blood lymphocytes (6) and nonreactive skin test reactions of *T. pallidum* antigen(s) (reviewed in reference 10). Furthermore, a more or less severe depletion is found in the thymus-dependent paracortical areas of lymph nodes in patients with early syphilis (7). It is generally accepted that a normal and efficient antibody production requires the cooperation between B and T lymphocytes. Disturbances in the T-cell branch could result in either a qualitatively or quantitatively altered T helper function, possibly leading to a deviation in immunoglobulin synthesis, especially in the IgG class, which is the most T-cell dependent. In immunological studies of syphilis these considerations so far seem to have escaped attention, and this instigated the present investigation on aspects of IgG synthesis in this particular disease. It was anticipated that such studies would also reveal a disturbed humoral antibody response suggestive of a deviation in intercellular mechanisms underlying antibody production, and also open possibilities for an in vitro experimental approach of this impaired immune system.

As previously reported from our laboratory, increase in serum IgG level in syphilis patients occurred to a large extent in the most basic part of electrophoretically heterogeneous IgG (14, 15). The availability of techniques to isolate IgG fractions of different electrical charge and access to various serological procedures to delineate the occurrence of antitreponema antibodies presently enabled us to examine the antitreponema antibody activity in IgG fractions of different charge and to investigate whether this antibody activity had the same preference for the basic part of heterogeneous IgG.

MATERIALS AND METHODS

Sera were obtained from 33 untreated male patients, aged 19 to 45 years (average, 28 years). In all patients the clinical diagnosis of syphilis, carried out by the method described by Menke (2; Menke, Ph.D. thesis), was confirmed by positive dark-field examination, or positive *T. pallidum* immobilization reaction, or both. Seventeen of the patients had primary syphilis; nine of these were in the serochanging stage ($S1^{\pm}$), and eight were in the seropositive stage ($S1^{+}$). Nine patients had secondary syphilis (SII), and seven had latent syphilis with a duration of infection shorter than 2 years (early latent syphilis, ELS). Case histories of the latter patients indicated that they most likely had passed the secondary stage. Patients in whom concomitant disease was diagnosed were excluded from the study.

Sera were stored at −70°C until use. IgG was iso-

lated from the sera by ion-exchange chromatography on diethylaminoethyl-Sephadex A-50 (Pharmacia, Uppsala, Sweden) equilibrated in 0.015 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.6, starting buffer). The bed volume of the column (K16/40, Pharmacia, Uppsala, Sweden) consisted of 70 ml. One batch of diethylaminoethyl-Sephadex A-50 was used throughout the experiments described. Ten milliliters of serum, dialyzed against 100-fold excess of the starting buffer for 48 h, was pipetted on top of the Sephadex bed. Next, discontinuous gradient elution was performed with three tris(hydroxymethyl)aminomethane-hydrochloride buffers. The first fraction (fraction 1) was eluted with the starting buffer and consisted of the unretarded material, fraction 2 was eluted with 0.020 M tris-(hydroxymethyl)aminomethane-hydrochloride ✔ (pH 7.5), and fraction 3 was eluted with the same buffer at 0.030 M and pH 6.0. The amount of protein present in each fraction was calculated from its volume and the protein content, determined spectrophotometrically from the extinction at 280 nm using human IgG as a standard. Fractions were concentrated in Amicon concentrating cells equipped with PM 10 membranes (Amicon, Oosterhout, The Netherlands). Thereafter, the purity of fractions was examined by the double-immunodiffusion method of Ouchterlony, using specific antisera against serum IgA, IgM, and IgG and antiserum against human serum (Central Laboratory of the Dutch Red Cross, Amsterdam, The Netherlands). The relative electrophoretic mobility of the proteins of the various fractions was assessed by using the electrophoresis technique of Wieme (19). To this end, fractions containing 5 mg of eluted protein per ml were made 1 mg/ml in human albumin (Behringwerke Chemie, Marburg/Lahn, lot 3566) and 1 mg/ml in human transferrin (Behringwerke Chemie, lot 2469D).

These proteins served as reference proteins to determine the point of zero mobility by scanning electropherograms on a densitometer (15). For each fraction, the distance between its point of highest density to the zero mobility point was expressed as a percentage of the total length of the electropherograms.

Before quantitation of antibody activity in the various fractions, they were dialyzed against 0.01 M phosphate-buffered saline (pH 7.0) and subsequently diluted to 1 mg/ml in phosphate-buffered saline. Next, quantitation was performed by applying, for each fraction, four serodiagnostic tests for syphilis, carried out as described by Veldkamp and Visser (16): (i) the Reiter complement fixation reaction; (ii) the fluorescent treponema antibody-absorbed reaction; (iii) the enzyme-linked immunosorbent assay, modified for syphilis; and (iv) the Wassermann-Kolmer reaction. The complement inactivation step in the complement fixation reactions (i and iv above) was omitted, since it was shown to cause anticomplementarity of the fractions.

In each test spontaneous complement consumption was checked, and, if found, the titers of the complement fixation reactions were corrected for the anticomplement titer. The fluorescent treponema antibody-absorbed reaction was titrated in 1:12, 1:30, and 1:60 dilutions and further in 60-fold dilution steps, until a negative reaction was obtained. For the statistical analysis of the results, the Wilcoxon nonparametric test was used with the level of significance set at $2\alpha = 0.05$.

RESULTS

Double-diffusion experiments with appropriate antisera showed that each fraction contained IgG, but that IgA and IgM were not demonstrable. Using antiserum against human serum, the findings indicated the presence of only one serum protein. Thus, it may be concluded that in the isolated fractions no serum proteins other than IgG were demonstrable.

Relative mobilities of the fractions are given in Table 1. The Table demonstrates a distinct difference in electrophoretic mobility between the three fractions, fraction 1 showing the slowest mobility and hence containing more basic IgG molecules than the more "rapid" fractions, 2 and 3.

Average amounts of IgG eluted from the column and average amounts collected in the three fractions are given in Table 2. The only significant difference in the amount of IgG in comparable fractions, as revealed by statistical analysis, is between fraction 1 in the serochanging stage ($S1^{\pm}$) and fraction 1 in the secondary stage (SII).

Results of serological testing of the fractions containing 1 mg of IgG per ml are given in Table 3. A striking conclusion from the Table is that, of the three fractions obtained from serum of one particular patient, fraction 1 possessed a higher antibody activity than fraction 3. This was the case in all patients and was independent of the serological reaction used to delineate the presence of syphilitic antibodies. Fraction 2 as a rule showed intermediate activity. Differences

TABLE 1. *Mean distances of fractions 1, 2, and 3 to the point of zero mobility, expressed as a percentage of the total length of electropherograms*

Fraction	Mean distance	Standard deviation	*n*
1	3.6	2.6	20
2	10.5	1.9	20
3	18.8	1.3	19

TABLE 2. *Total amounts of IgG eluted from the columns and the amounts collected in the fractions*

IgG	Amt of IgG collected (mg)[a]			
	$SI^{\pm}$	SI^{+}	SII	ELS
Total	75 (20)	84 (27)	85 (22)	82 (23)
Fraction 1	22 (7)	27 (11)	32 (10)	30 (9)
Fraction 2	25 (9)	31 (16)	29 (9)	26 (13)
Fraction 3	28 (7)	26 (5)	25 (6)	27 (9)

[a] Means and (standard deviations).

TABLE 3. *Reactivities of IgG fractions*[a]

Stage	Patient no.	RPCF[b]			FTA-ABS[b]			ELISA[c]			WaK[b]		
		Fr. 1	Fr. 2	Fr. 3	Fr. 1	Fr. 2	Fr. 3	Fr. 1	Fr. 2	Fr. 3	Fr. 1	Fr. 2	Fr. 3
S1±	1	2	—	—	—	—	—	0.220	0.000	0.000	—	—	—
	2	2	1	1	12	12	—	0.607	0.323	0.094	1	1	—
	3	4	2	1	60	12	—	0.382	0.164	0.070	2	2	—
	4	1	—	—	—	—	—	0.103	0.086	0.072	2	—	—
	5	1	—	—	—	—	—	0.082	0.011	0.008	—	—	—
	6	2	2	1	—	—	—	0.071	0.024	0.014	—	—	—
	7	1	1	—	—	—	—	0.015	0.000	0.000	—	—	—
	8	4	2	—	30	—	—	0.550	0.610	0.350	4	4	—
	9	8	2	2	30	12	—	0.476	0.346	0.332	4	4	1
S1+	10	2	1	—	30	12	—	0.230	0.069	0.010	2	2	—
	11	8	2	—	120	60	30	1.660	1.140	0.413	4	2	1
	12	8	2	—	120	120	30	1.090	0.870	0.292	16	2	—
	13	8	4	2	120	60	12	0.551	0.442	0.079	4	2	1
	14	4	1	1	30	—	—	0.609	0.482	0.149	1	—	—
	15	4	2	2	360	120	60	0.390	0.188	0.115	4	4	1
	16	16	8	4	360	240	60	0.790	0.660	0.288	16	8	1
	17	4	2	1	60	60	30	0.616	0.130	0.000	8	8	2
SII	18	16	8	4	120	30	12	1.260	1.040	0.372	8	8	2
	19	32	8	8	720	360	120	2.940	2.470	1.160	4	8	2
	20	4	1	—	120	30	—	1.041	0.617	0.505	2	1	1
	21	16	8	2	240	120	12	1.880	1.470	0.645	16	16	4
	22	2	2	1	120	120	60	0.884	0.699	0.309	4	2	1
	23	2	1	1	120	60	30	0.428	0.220	0.106	4	2	2
	24	8	2	1	60	30	12	0.830	0.620	0.106	4	4	1
	25	32	16	4	720	360	360	1.740	1.400	1.010	8	8	2
	26	8	4	4	480	540	120	1.300	1.160	0.920	8	8	2
ELS	27	2	1	1	30	12	—	1.050	0.846	0.448	2	2	1
	28	8	4	4	300	180	60	2.560	2.220	1.340	8	16	4
	29	2	2	—	30	30	12	1.020	0.820	0.830	4	2	—
	30	4	4	1	480	420	60	1.540	1.280	1.200	4	4	4
	31	8	4	4	780	660	480	2.120	1.910	1.720	8	8	4
	32	8	8	2	480	120	120	1.400	1.140	0.538	4	4	4
	33	16	8	4	480	360	240	1.170	1.360	1.070	4	8	4

[a] Initial IgG concentration: 1 mg/ml. RPCF, Reiter complement fixation reaction; FTA-ABS, fluorescent treponema antibody-absorbed reaction; ELISA, enzyme-linked immunosorbent assay; WaK, Wassermann-Kolmer reaction.

[b] Figures indicate the highest dilution in which a positive reaction was found. —, Negative reaction.

[c] Figures represent the extinctions found after correction for the blank.

in antibody activity between fractions 1 and 3, however, were less pronounced in the ELS patient group when the Wasserman-Kolmer reaction, which detects antilipoidal antibodies, was used.

A combination of present findings on electrophoretic mobilities with those of antibody activities indicates that antibody activity expressed on the basis of the same amount of IgG is largest in the most basic part of heterogeneous IgG and decreases in more acidic parts.

A second conclusion to be drawn from Table 3 concerns the sequence in which antibody activity arises in the fractions in the course of the disease. In fractions from sera of some patients in stage S1±, no antibody activity could be detected in one or more of the serological tests used. In some other, comparable patients, either fraction 1 was the only fraction in which antibody activity could be detected or it contained the highest activity. As compared to the S1± stage, the antibody activity in all three fractions increased while the disease was passing through the S1+ and SII stages. During this process fraction 1 generally retained the highest antibody activity. This is also apparent from Table 4, which gives mean titers of antibody activity. From this Table it would appear that the relative differences between the fractions are highest during the SI± and the SI+ stages and tend to diminish during SII and ELS stages. Particularly in the SII stage, this is not due to a decrease of

TABLE 4. *Mean titers of IgG fractions (Fr.)*[a]

Stage	RPCF[b]			FTA-ABS[b]			ELISA[c]			WaK[b]		
	Fr. 1	Fr. 2	Fr. 3	Fr. 1	Fr. 2	Fr. 3	Fr. 1	Fr. 2	Fr. 3	Fr. 1	Fr. 2	Fr. 3
$SI^{\pm}$	2.8	1.1	0.6	15	4	0	0.278	0.174	0.104	1.4	1.2	0
SI^{+}	6.7	2.8	1.3	150	84	28	0.742	0.498	0.168	6.9	3.5	0.75
SII	13.3	5.6	2.8	300	183	81	1.367	1.077	0.570	6.4	6.3	1.9
ELS	6.9	4.4	2.3	368	255	139	1.551	1.368	1.021	4.9	6.3	3.0

[a] Initial IgG concentration, 1 mg/ml. RPCF, Reiter complement fixation reaction; FTA-ABS, fluorescent treponema antibody-absorbed reaction; ELISA, enzyme-linked immunosorbent assay; WaK, Wassermann-Kolmer reaction.

[b] See Table 3, footnote *b*.

[c] See Table 3, footnote *c*.

antibody contents of fraction 1, but to an increase of antibody contents of fractions 2 and 3. This was particularly clear when the Wassermann-Kolmer reaction was used for the SII and ELS patient groups. The Table shows that differences between fractions 1 and 2 were no longer existent or were reversed.

DISCUSSION

In comparing present results of serological tests of corresponding fractions of sera from syphilis patients, a uniform way of reacting to different treponemal and lipoidal antigens emerges. Fraction 1 shows the highest antibody activity, which gradually diminishes in fractions 2 and 3. Electrophoresis of the proteins in the various fractions revealed that the column efficiently separates fractions of different relative mobilities. This would indicate that the basic IgG contributes to a large extent to the overall antibody activity in the IgG class. This is supported by the sequence of detection of antibody activity in the various fractions. Previously (15) we have shown that after infection with *T. pallidum*, the increase in serum IgG should be attributed to a large extent to the (arbitrarily) defined basic IgG. This is in accordance with the present finding of a significant larger amount of IgG, collected in fraction 1 in stage SII as compared to fraction 1 in stage $SI^{\pm}$. Thus, the basic part of heterogeneous IgG may make a large contribution to the overall antibody activity in the IgG class because of (i) high "specific activity" and (ii) increase after infection. This results in an asymmetric profile of antitreponemal antibody activity.

Several explanations of our findings can be offered, although they are all more or less speculative. One possibility could be that our observations are a consequence of a restriction of antitreponema antibodies to the IgG1 subclass, as described by Puritz et al. (11). Howard and Virella (5) found that at the cathodal site the IgG1 subclass proteins extend to somewhat higher isoelectric points than do IgG2 and IgG3 proteins. We separated our fractions on ion-exchange columns. Since this is generally accepted to occur mainly on the basis of different electrical charge, a certain preference for IgG1 in fraction 1 seems probable. This would explain the higher antibody content of fraction 1 as compared to fractions 2 and 3. The IgG1 present in the latter fractions will be progressively diluted with the "silent" subclasses 2 and 3, and these fractions will show, by consequence, a lower antibody content. However, our studies on subclass levels in serum of syphilis patients and on antibody content of the various subclasses (J. J. van der Sluis, E. C. van Reede, and M. Boer, manuscript in preparation) do not confirm the above-mentioned restriction found by Puritz et al.

A second possibility is an explanation of our observations on the basis of an inverse charge relationship mechanism. Sela and Mozes (12) have shown in mice that acidic antigens elicit basic antibodies and vice versa. A similar inverse charge relationship has been shown to be possible in the human immune system (13). Our present results could possibly be interpreted as being the expression of the acidic character of (major) treponemal antigens. However, some objections can be put forward against this explanation. First, although very little is known about the nature of treponemal antigens, it seems reasonable to expect that *T. pallidum* will give rise to a cocktail of antigens which will, most likely, differ in physicochemical characteristics. In our opinion, it would be surprising if such an antigen cocktail were to lead to the observed uneven distribution of antibody activity and, more important, to an uneven distribution of de novo-synthesized IgG, as described previously (15). Second, it has been shown in normal mice that the inverse charge relationship exists only for T-dependent antigens (4). Consequently, its demonstration implicates the presence of a proper functioning T-cell branch. In humans (6, 10), as

well as in rabbits (8, 9, 17, 18), there are indications of an impairment of the T-cell branch early in syphilitic infection. When (in humans) the expression of the inverse charge relationship is governed by the same factors as it is in the murine immune system, the impairment of the T-cell branch could invalidate the explanation of our observations on the basis of an inverse charge relationship.

A third explanation could be that the present results are the consequence of an improperly functioning T-cell branch. Since activated T cells or their products are able to regulate B-cell responses, impairment of T-cell function might influence antibody production during early syphilis. Indeed, Baughn and Musher (1) have shown that in spleens of syphilitic rabbits, production of indirect plaque-forming cells to the unrelated T-dependent sheep erythrocyte antigen is strongly depressed early in infection and returns to normal after overt infection. In these rabbits, this depression is reflected in low levels of hemagglutinating IgG antibodies to sheep erythrocytes as compared to uninfected animals.

Particularly in the earliest stages ($SI^{\pm}$ and SI^{+}), our results show a low antibody content in fraction 3 and an intermediate level in fraction 2 in comparison to fraction 1. In connection with the findings of Baughn and Musher in rabbits, our results could point to a depressed IgG synthesis during early syphilis in humans. The gradual increase in antibody content of fraction 2 and 3, which happens, especially in the latest stages studied, at a higher rate than the increase in fraction 1, might be regarded as a first sign of IgG synthesis returning to normal. This would coincide with the stage at which positive skin test reactions first emerge during the course of human syphilis. Which part is played by each of the possible mechanisms mentioned remains to be studied.

Our observations show a bimodal development of IgG antibody response to a variety of antigens in early syphilis: a rapid development in the (small) most basic part of heterogeneous IgG and a slow development in the remaining part. Although no information is available on the quality of basic and acidic antitreponemal antibodies, the slow development of the acidic IgG antibodies might bear some relationship to the fact that humoral immune response in early syphilis is not protective.

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