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Separation of Antibodies from Subcellular Components of Lymphocytes

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Summary. Soluble supernatant fluids obtained from disrupted human lymphocytes were fractionated by gel filtration and ion exchange chromatography. Three populations of antibody molecules were obtained. One of these was a fragment of γG globulin; the remaining two were γG globulins immunologically indistinguishable from serum γG globulins. Of these last two populations, one was associated with residues of ribonucleic acid; the other with residues of deoxyribonucleic acid. The latter was linked by a disulphide bond to an acidic acceptor protein; the acceptor in turn appeared chemically bound to nuclear DNA. Uptake of antigen by lymphocytes occurred only when antibody was bound to the acceptor (activated antibody).

INTRODUCTION

Human lymphocytes contain γG globulin molecules which possess antibody activity for antigens to which the cell donors are immune (Merler and Janeway, 1968). In the accompanying communication, evidence is presented which shows that these cell-bound antibodies are present in those cells that interact with and take up antigen (Merler and Silberschmidt, 1972).

The experiments described herein present evidence that at least some cell-bound antibody is chemically linked to an acceptor protein which in turn is bound to nuclear DNA.

MATERIALS AND METHODS

Separation of lymphocytes and fractionation of cells. Lymphocytes were separated from tonsils and adenoids of children who had been immunized against diphtheria and tetanus toxoid. Cells were freed from the tissues, washed, and fractionated on discontinuous density gradients of bovine serum albumin (BSA) (Dicke, vanHooft and vanBekkum, 1968). Concentration limits of 35 per cent and 17 per cent BSA were employed. Following centrifugation, cells were counted in a Coulter counter. They were extensively washed in medium 199 (without serum).

Cell disruption and fractionation of soluble supernatant fluids. Cell pellets were suspended in ten volumes of distilled water and sonicated at 50 watts (30–60 seconds at 2°). Solutions

were made 0·1 M with KCl and digested with deoxyribonuclease I (Worthington Biochemicals—1× crystallized) in $MgCl_2$ (10^{-2} M) at 37° for 6 hours. Undigested, high molecular weight nucleic acids were rendered insoluble by salt (final concentration 0·15 M) and sedimented by centrifugation at 150,000 ***g*** for 30 minutes. Components having different molecular weights were separated (Table 1) from one another by gel filtration on columns of Sephadex G-50 or G-100 resin (2·5×100 cm) (Pharmacia Fine Chemicals) and agarose A50 (0·9×100 cm) (Bio-Rad Laboratories) by ascending chromatography; components having different charge distributions were separated on columns of O-(diethylaminoethyl) [DEAE] cellulose in a buffer of tris(hydroxymethyl) amino methane (tris) and HCl (pH 8·2, μ 0·012). Salt gradients were formed with LiCl (2 M) (Traub and Nomura, 1968). Occasionally, separations on DEAE cellulose and the following salt gradients were done in buffers equilibrated in 8 M urea.

Digestion with ribonuclease (Worthington Biochemicals, 5× crystallized) were done in an ammonium bicarbonate buffer (μ 0·1) at pH 7·2 and 37° for 60 minutes.

Miscellaneous procedures. Determinations of antibody titre were done by measuring the haemagglutination of sensitized erythrocytes which had been treated with tannic acid followed by the antigen under study. Protein solutions at a concentration of 1 g of protein percent were assayed by doubling their dilution until an end point was reached. Proper controls were employed (Levine, Wyman, Broderick and Ipsen, 1960).

Quantitative γG globulin determinations were done by radial immunodiffusion in agar using commercial plates but laboratory-prepared standards (Mancini, Carbonara and Heremans, 1965).

Concentration of protein was measured in a synthetic boundary cell in the analytical ultracentrifuge (Richards and Shachman, 1959) or alternatively by the phenol reaction described by Lowry, Rosebrough, Farr and Randall (1951).

Digestion with proteolytic enzymes were done at a substrate to enzyme ratio of 100:1.

Digestions with subtilisin (Nagarse) were done at pH 8·0 in an unbuffered solution in the pH stat.

RESULTS

For the purpose of chemical fractionation, cells obtained from the BSA gradients were divided into two major classes. Those contained in layers 1–4 (inclusive), which were large, mitotically active lymphocytes, were pooled in one fraction (L); and those contained in layers 5–7 (inclusive), which were small, mitotically stable lymphocytes, into another (S). The scheme of fractionation used is shown in Table 1.

Fractionation on a G-100 resin of a 150,000 ***g*** supernate obtained from cells dispersed in water by sonication is shown in Fig. 1. Fractions FLI and III (Fig. 1) reacted with an antiserum raised in rabbits to human serum γG globulin. In two dimensional gel diffusion, fraction FLI gave a reaction of complete identity with serum γG globulin; while FLIII gave a reaction of partial identity with spur formation, indicating that the former fraction contained γG globulin immunologically indistinguishable from that found in serum, while the latter fraction contained fragmented γG globulin.

The pattern shown in Fig. 1 was obtained from a supernatant of cells that had not been fractionated on BSA gradients. In general, both fractions S and L from the BSA gradient produced FLI, but only L gave FLIII. FLI, which was excluded from the Sephadex resin,

TABLE 1

FRACTIONATION SCHEME OF SUBCELLULAR COMPONENTS WHICH CONTAIN ANTIBODY, AND SEPARATION OF A PEPTIDE

1. Cell supernatant

fractionated on Sephadex G-100

FL II–V

FL 1

fractionated on agarose A50

FL 100 1 A 2, 3

FL 100 1 A 1

fractionated on DEAE cellulose

low salt, FL 100 1 A 1 X

High salt, FL 100 1 A 1 Z

fractionated on DEAE cellulose in MEA

low salt, G

High salt, A peptide

was further fractionated into three fractions (1, 2 and 3), all included on a column of agarose A50 (Fig. 2).

Only fraction I (FL 100 I A 1) (Fig. 2) reacted with the antiserum to human serum γG globulin. This fraction was applied to a column of DEAE cellulose. Several fractions were obtained (Fig. 3), only two of which (X and Z) reacted with the antiserum to γG globulin. X was eluted from the DEAE column at low salt concentration, while Z was eluted at high salt concentration. While X and Z were not exclusively derived from the two original

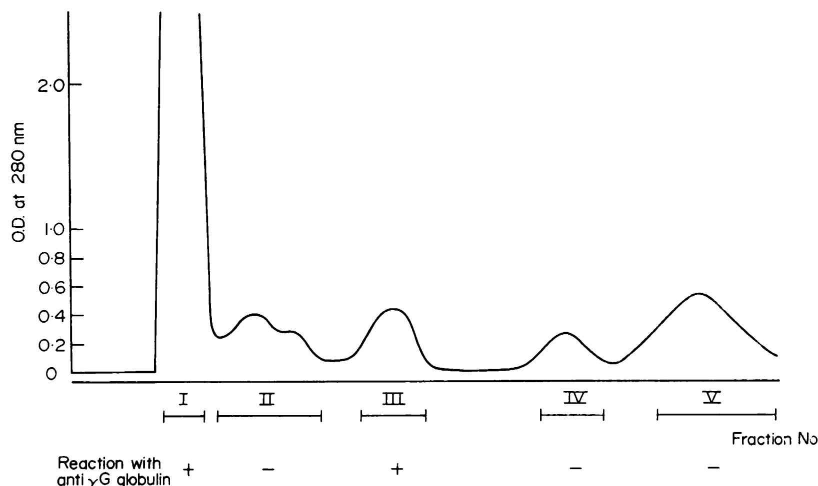

FIG. 1. Fractionation on 0·15 M NaCl of a supernate obtained from sonicated lymphocytes (FL) on a column of Sephadex G-100 resin: optical density at 280 nm vs. fraction number. Five fractions were obtained indicated by numerals along the axis of abscissa. The reaction of these fractions with an antiserum to human serum γG globulin is indicated. The fractionation was repeated with similar results on at least ten different occasions.

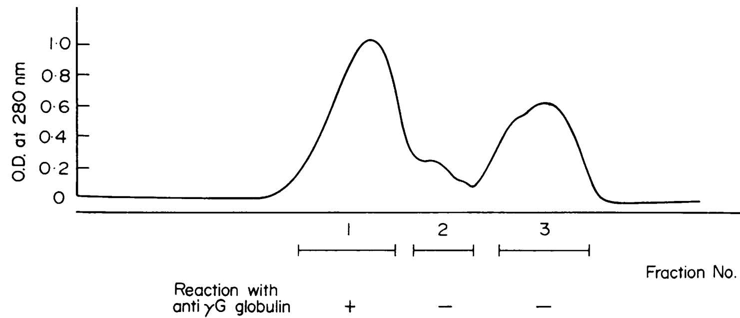

FIG. 2. Fractionation in 0·15 M NaCl of FL 100 I on a column of agarose A50: optical density at 280 nm vs. fraction number. The reaction of these fractions with an antiserum to human serum γG globulin is indicated. The fractionation was repeated with similar results on ten different occasions.

BSA fractions L and S respectively, L produced principally X, while S gave Z. Fraction Z, adsorbed on DEAE cellulose at low salt concentrations in 8 M urea, eluted in a salt gradient in 8 M urea as a single component at a salt concentration similar to that which eluted it in the absence of urea.

The fraction eluting at low salt (FL 100 I A 1 X) (Fig. 3) had a ratio of optical density readings in the spectrophotometer at 260 and 280 nm of 1·2 and showed in the centrifuge a single boundary having a sedimentation coefficient $S_{w,20}$ 6·1. This S value remained unchanged after treatment of this material with deoxyribonuclease II, but was increased to 7·2 after treatment with ribonuclease. Digestion with ribonuclease released dialysable, U.V.-adsorbing materials and concomitantly changed the 260/280 ratio to 0·6. These results suggest that the γG globulin may be bound to a ribonucleic acid residue, which was digested by the RNAase. The increase in S value possibly represents changes of the polymer to a globular conformation following digestion of the RNA residues.

The fraction eluting with high salt (FL 100 I A 1 Z) (Fig. 3) had a 260/280 ratio of 1·4 and an S value of 13. The S value remained unchanged after digestion with ribonuclease

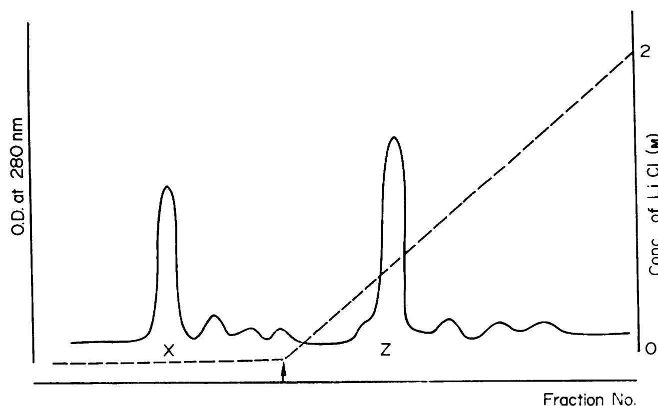

FIG. 3. Fractionation of FL 100 I A 1 on DEAE cellulose: optical density at 280 nm vs. fraction number. Starting buffer: Tris/Hcl 2×10^{-2} M pH 8·3. At the point indicated by an arrow, a linear gradient was started in which the limits were the starting buffer and a buffer of NaAc/HAc 10^{-2} M pH 4·5 in 2 M LiCl. The fractionation was repeated with similar results on five different occasions.

but was changed to 8·0 following digestion with deoxyribonuclease II. These results suggest that fraction Z was bound covalently to residues of DNA since urea failed to dissociate the complex but DNAase did. After digestion with deoxyribonuclease, immunological quantitation of Z with an antiglobulin serum indicated that only 65 per cent of the digest reacted with the antiglobulin. After digestion with deoxyribonuclease, Z contained a chemically bound polymer that was not γG-globulin. Fraction FL 100 I A 1 Z was applied to a column of DEAE cellulose and separated under conditions identical with those depicted in Fig. 3 with the exception that 6×10^{-3}M mercaptoethanol (MEA) was added to the buffers (Fig. 4).

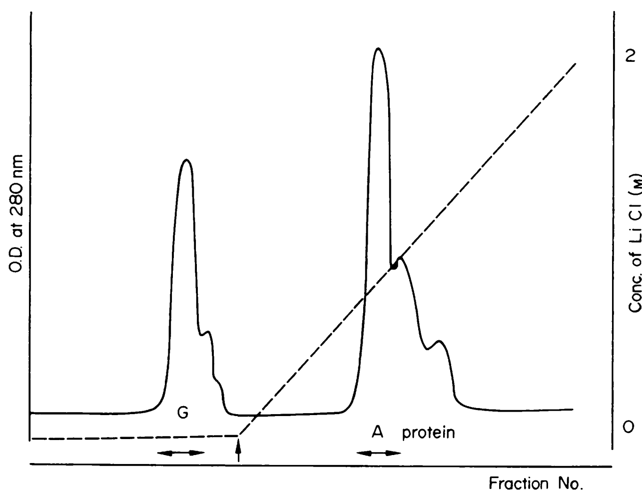

FIG. 4. Fractionation of FL 100 I A 1 Z on a column of DEAE cellulose: optical density at 280 nm vs. fraction number. Starting buffer: Tris/HCl 2×10^{-2} M, pH 8·3, in 6×10^{-3} M MEA. The gradient applied at the arrow point reached a limit with a buffer of NaAc/HAc 10^{-2} M, pH 4·5, in 2 M LiCl and 6×10^{-3} M MEA. The fractionation was repeated with similar results on five different occasions.

Two major peaks of protein were eluted. The material (G) eluting at a low salt concentration with the starting buffer had a 260/280 ratio of 0·6 and a sedimentation coefficient $S_{w,20}$ 6·8 which remained unchanged after treatment with deoxyribonuclease II. It reacted with the antiserum to serum γG globulin to give a reaction of identity to that obtained with serum γG globulin; furthermore, γG was precipitated quantitatively by the antiglobulin serum. It was therefore concluded that peak 1 (G) was free γG globulin. The material eluting at high salt from the DEAE column had a 260/280 ratio of 1·4 and S value of 4·2. It did not react with the antiserum to γG globulin. After hydrolysis with deoxyribonuclease II and separation of the nucleotides on a column of Sephadex G-25, the material excluded from the resin (A peptide) had a 260/280 ratio of 0·6 and S value of approximately 1. The amino acid composition of A peptide is shown in Table 2. Hydrolysis of A protein with subtilisin followed by fractionation of the peptides on a column of acrylamide P_2 gel yielded two peaks, the first having a 260/280 ratio greater than 1 and the second smaller than 1.

Acid hydrolysis of peak 1 yielded equal amounts of the amino acids aspartic acid and valine, plus several unidentified ninhydrin-negative but UV-adsorbing components.

TABLE 2

AMINO ACID COMPOSITION OF A PEPTIDE

Amino acid	No. of residues*
Cys	1
Asp	5
Thr	3
Ser	4
Glu	7
Gly	14
Ala	4
Val	3
Ile	1
Leu	2
Tyr	2
Phe	2
Lys	4
His	2
Arg	1
Unk	3

* Presumptive molecular weight of A protein was assumed to be 6200, obtained by adding the molecular weight of each residue (replicate analyses). This assumption was justified by a sedimentation equilibrium run which gave a molecular weight of 7000 ± 1000.

These were identified both by quantitative amino acid analysis and by high voltage electrophoresis at pH 1·9. Peak 2 after acid hydrolysis yielded a mixture of amino acids, qualitatively similar to that obtained by acid hydrolysis of the A protein (high voltage). Peak 1 was interpreted as containing a peptide that was bound to DNA residues. Antibody titres of fraction G were 2 and 3 haemagglutinating units per mg; those of fraction Z were 0·4 units per mg.

These experiments show that in lymphocytes γG globulin may either occur bound to RNA (in L cells), presumably on the polyribosomes (FL 100 I A 1 X), or bound to DNA (in S cells) (FL 100 I A 1 Z). In S cells, however, the antibody does not appear bound directly to DNA but rather to an acidic protein (A). The A protein, like a bridge, is bound to antibody at one end by an easily reducible disulphide bond, and to DNA at the other by a chemical bond presumably between an aspartic acid and deoxyribonucleotides.

DISCUSSION

In the present communication, an attempt has been made to define the role of cell-bound antibody in the process of antigen uptake by studying the localization of antibody in the lymphocyte. From these studies, the S population, made up of dense, non-dividing cells which take up antigen, appears the more interesting.

Since the antigen following uptake by the cell does not remain bound to the cell surface of the lymphocyte but instead 'migrates' to the nucleus (as shown in the following paper: Merler and Silberschmidt, 1972), it appears logical that, to accumulate antigen, the cell should have a mechanism for trapping it in the nucleus. Two questions arise. Since the cells that take up antigen do not divide, why do they need to accumulate antigen; and secondly, if antibody mediates recognition, why are antigen–antibody complexes never

found following antigen uptake when the cells are disrupted? Would such complexes be useful for expressing the potential of the cell? At best, only partial answers can be given to these questions. Preliminary evidence indicates that interaction of antigen with the immune cell releases a mitogen; it is not known if the amount of mitogen released is proportional to the concentration of antigen in the cell, nor if release of mitogen is the only function of the immune S lymphocyte. If, however, more mitogen were released by larger amounts of antigen taken up by the cell, accumulation of antigen in the cell would be desirable. Secondly, the A peptide (acceptor protein), a small molecular weight acidic peptide bound to both antibody and nuclear DNA, appears to play a crucial role in defining the activity of cell-bound antibody and, in a broader sense, of the competent cell. Passively supplied antibody, although cell bound, was never found joined to the acceptor; that is, antigen uptake occurred only in the presence of antibody bound to the acceptor ('activated antibody'). Interestingly, recovered antibody reacted freely with antigen to give a haemagglutination reaction when separated from the acceptor but appeared relatively unreactive by exhibiting a considerably smaller haemagglutination titre when bound to the acceptor. The significance of this can only be surmized, but it seems to indicate that it is not the function of antigen to bind with antibody to give a precipitin reaction in the cell and, therefore, that it is not the complexing of antigen with antibody that activates the immune process. It has also been reported that antigen does not act as a direct template on polysomes for the formation of antibodies in plasma cells (Nossal, Ada and Oustin, 1965). Antigen could, however, have a regulatory effect in the transfer of immunological information at the nuclear level.

Free acceptor protein unbound to antibody is separable from the cell nuclei; by incubating this free acceptor with antibody *in vitro* in the presence of either intact mitochondria or cell supernatants, we have been unable to form a complex similar to that separated from the cell, although scant evidence for the formation of molecular aggregates was found by these experiments. Immune cells appeared to contain only antibodies reactive to the immunizing antigens, suggesting that activated antibody was acquired by the S cells following immunization.

Finally, mention should be made of the other two populations of antibody molecules, those that are found in the dividing L lymphocytes, the antibodies bound to ribonucleotides, and the fragmented antibodies. While studies are in progress to characterize chemically the fragmented antibodies, no reason is apparent for their physiological role, save that of being precursors of complete antibodies. These fragments do not appear bound to ribonucleotides. The cells containing these two populations of antibodies are not plasma cells and cannot consequently be involved in active secretion. It is suggestive, therefore, that here again cell-bound antibodies may be regulators of some cellular activity although their role must at present remain unknown.

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