FURTHER STUDIES ON THE ULTRASTRUCTURE OF
DIMERIC IGA OF HUMAN ORIGIN*

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We have previously reported on the architecture of secretory IgA (s-IgA)¹ protein molecules (1) using electron microscopy and the conventional technique for negative staining described by Brenner and Horne (2). A modification of this technique, the so-called mica technique, was introduced by Valentine (3). The mica technique has been found particularly useful in studies of certain macromolecules such as immunoglobulins (4, 5) which are difficult to distribute evenly on a hydrophobic carbon film by the conventional method. The aim of the present investigation was to procure further ultrastructural information on dimeric human IgA and to compare the merits of the mica technique with the conventional method for negative staining.

Materials and Methods

Purification of IgA.—Secretory IgA from human colostrum, collected within 24 hr after delivery, was purified by a combination of ultracentrifugation and gel filtration chromatography on a tall (2.5 X 400 cm) Bio Gel A 15 m column (Bio-Rad Laboratories, Richmond, Calif.) as earlier described (1). Five different human colostral IgA preparations were prepared and examined.

Serum from a patient with IgA myeloma² was fractionated by a two-step procedure. 10 ml of serum was gel filtered on the same tall Bio Gel column as was used for purification of the s-IgA material. Tubes containing high polymer IgA, based on immunodiffusion analyses and position on the elution curve, were concentrated by vacuum dialysis. This material also contained some β-lipoprotein, α2M globulin, and haptoglobin. These contaminating components were removed on an immunoadsorbent column by polymerizing a protein solution (50 mg/ml) containing specific antibodies against these contaminants. The protein solution was dialyzed for 24 hr against 0.15 M NaCl buffered with 0.01 M phosphate, pH 7.2 (PBS), and polymerization was performed by adding glutaraldehyde (TAAB, Reading, England) at a final concentration of 0.05%. One part of the polymer was homogenized in an omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) and mixed with 9 parts Sepharose 4B (Pharmacia Fine Chemicals,

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1 Abbreviations used in this paper: PBS, phosphate-buffered saline; S. C., secretory component; s-IgA, secretory IgA.

2 Kindly supplied by Dr. Folke Lindström, Lund, Sweden.
Inc., Uppsala, Sweden) in a 1.4 X 8 cm column. After 1½ hr absorption to the immunoadsorbent polymer, the IgA-containing material was eluted with PBS at a flow rate of 2.5 ml/hr and cm² and was tested for purity by immunodiffusion.

Reduction and Alkylation.—Purified human s-IgA was reduced in 0.08 M β-mercaptoethanol (Fluka AG, Basel, Switzerland) and alkylated in 0.001 M iodacetamide (Sigma Chemical Co., St. Louis, Mo.) as described by Brandtzaeg (6). The reduced and alkylated material was fractionated on a Sephadex G-200 column (Pharmacia Fine Chemicals, Inc.) equilibrated with 0.5 M NaCl buffered with 0.1 M tris(hydroxymethyl) aminomethane-HCl, pH 8.0, plus 2% n-butanol at 4°C. The material in the void volume and in the second peak was further analyzed by immunodiffusion and in the electron microscope.

Protein Estimation.—Protein concentrations were measured by absorbance at 280 nm in a 1.0 cm quartz cuvette with a Beckman DU spectrophotometer arbitrarily using an extinction coefficient (ε = ε₀λ) of 13.9 in all calculations.

Immunodiffusion.—Double diffusion analyses were performed on glass slides in 1% agarose (Bio-Rad Laboratories) gel and PBS, pH 7.2. Specific antisera to human serum proteins were purchased from Behringwerke AG, Marburg-Lahn, West Germany. Rabbit anti-colostral whey sera were produced in our laboratory.

Analytical Ultracentrifugation.—Purified IgA samples at different concentrations were dialyzed against 0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl before centrifugation. The runs were performed in a Spinco model E ultracentrifuge at 52,620 rpm and 20°C using a 12 mm 4° sector cell with standard or wedge windows. The sedimentation coefficients were calculated by conventional methods using η ≈ 0.71.

Electron Microscopy.—The method of preparing the samples for electron microscopy was essentially that described by Green (7). A Philips EM 200 equipped with an anti-contamination device and a 50 μ objective aperture was used. Calibration of the magnification was performed with a carbon grating having 54,864 line/inch (Ladd Research Industries, Inc., Burlington, Vt.). All pictures were taken at an initial magnification of 48,750 at 60 kv and in through focal series. To enhance the contrast, some pictures were rephotographed with normal, fine-grain panchromatic film. A satisfactory distribution of the IgA molecules on the carbon film was achieved by mixing with a small quantity of ferritin molecules. (2 X crystallized, Pentex Biochemical, Kankakee, Ill.).

RESULTS

The s-IgA preparations at first sight seemed to contain molecules of a rather heterogeneous appearance. Examination of a great number of micrographs did, however, reveal an unambiguous predominance of slender, elongated molecules as seen in Fig. 1. When examined at higher magnification these molecules had a shape resembling two Y joined by the single bars (Fig. 2). Molecules shaped as a single Y were also seen (Fig. 3e) but showed no predominance as in the previous investigation (1) in which the staining technique of Brenner and Horne (2) was used. The distance between the two bifurcations of the dimer molecule, comprising the two Fc parts, was 145–155 Å and this central part had a width of 35–40 Å. The length of the arms at the bifurcations, here assumed to represent the Fab parts, was 65–75 Å and their width 30–35 Å. Many molecules showed a tendency of bending at a point in the middle of the molecules (Figs. 2 and 3). The angle between the two joined monomers was observed to vary from 180°, in the completely stretched-out molecules, to about 60°.
When myeloma IgA, eluted from the Bio Gel A 15 m column in the same position as s-IgA, was studied these preparations were found to contain the same type of basic structure as in the s-IgA samples (Fig. 4). Measurements of myeloma dimer IgA molecules indicated that the Fab parts had the same dimensions as in the s-IgA molecule. The values obtained for the length of the Fc region averaged around 140 Å, suggesting a slightly shorter Fc than in the s-IgA molecule.

The 7S material isolated by gel filtration on Sephadex G-200, after reduction and alkylation of human s-IgA, contained an abundance of molecules consisting of three bars joined in single Y forms (Fig. 5). Two of the appendages had the same dimensions as the two Fab parts in the intact s-IgA molecules and in high polymer myeloma IgA. The third arm, representing the Fc part, was 35–
FIG. 2. Electron micrographs of human colostral IgA molecules at high magnification. A certain degree of bending at a central point is visible in some of the molecules. × 245,000.

FIG. 3. Colostral IgA molecules showing different modifications of the basic double Y structure. Note the presence of a molecule visible as a single Y (arrow). × 245,000.

FIG. 4. Electron micrographs at high magnification of human myeloma dimer IgA. × 245,000.

FIG. 5. Electron micrographs of molecules from the second peak of a G-200 run of reduced and alkylated human colostral IgA. × 245,000.
40 Å in width and had a length of 65–70 Å. Molecules in the first eluted peak of the same G-200 run were morphologically undistinguishable from untreated 11S s-IgA molecules.

DISCUSSION

The s-IgA molecule may have a rather high degree of intramolecular flexibility as suggested by the molecular heterogeneity observed on the micrographs. The molecules could also have been distorted to a different extent by the various forces which come into play on the carbon film such as surface tension, vapor pressure, etc. A certain tendency to aggregation of the molecules, also adding to the heterogeneous appearance, was noted. Many s-IgA molecules were bent or twisted (Figs. 2 and 3) around a flexible region in the central part of the molecule, probably representing the joining point of the two monomers.

There are two alternative explanations for the existence of single Y-like molecules in the electron micrographs of purified s-IgA preparations. These structures may be true IgA monomers released by disruption of the dimer molecule. Alternatively, they can represent two superimposed monomers, which are still united, as was proposed in a previous paper (1). Such a superimposition may be permissible by the high flexibility of the bridging region. If the explanation for the previous observations (1), where single Y-forms were found to predominate in s-IgA preparations, was a dissociation of the intact dimer, it is hard to conceive how by a comparatively slight modification of the staining technique this dissociation could be prevented. It should also be recalled that IgM, α2M, and thyroglobulin showed no significant tendency to dissociate when the conventional Brenner and Horne staining technique was used (8–10). In an attempt to investigate whether the frequently seen Y-like particles actually represented monomers released from s-IgA, purified s-IgA preparations were dried onto glass slides in the presence of 1% uranyl oxalate and 1% uranyl formate, both at pH 4.6, or 2% sodium tungstosilicate, pH 6.0, using the conventional staining procedure (2). The material, containing approximately 5 mg protein/ml, was then redissolved in PBS and gel filtered on a small Bio Gel 1.5 m column (8 × 750 mm). Only one peak was visible on the chromatogram corresponding to the 11S peak from the untreated control s-IgA preparation.

The s-IgA molecule is known to contain an additional bonded polypeptide rich in carbohydrate (9–10%) and with a molecular weight of 55,000–80,000 (11–14). This polypeptide has been designated secretory piece or secretory component (S.C.) (15) and its exact mode of linkage to the IgA is a matter of debate. S.C. has several hidden determinants (6, 12, 16) and according to Lawton and Mage (17), who have performed enzymic degradation experiments on rabbit s-IgA, the S.C. is linked to the Fc part of the IgA. The present investigation has not been able to resolve the exact position of this component. Compaa-
tive measurements of myeloma dimer IgA and human s-IgA molecules suggested a slightly longer (5-15 A) Fc region in the s-IgA molecules. However, due to the inherent difficulties in making exact measurements of this often bent or twisted region, further studies are necessary to establish the significance of this suggested length difference. Presently, S.C. does not contribute substantially to the length of the s-IgA molecule. This is compatible with the findings which indicate that IgA dimers, with or without bound S.C., exhibit comparable size properties (12, 17) and with data suggesting that S.C. is attached to preformed dimer IgA molecules rather than to IgA monomers (18-20). We have failed to demonstrate any morphological alteration of the double Y dimer molecule due to the attachment of S.C. Dourmashkin has made similar observations. One possible explanation for this unexpected finding could be that the S.C. has a thin, filamentous shape. Compatible with this explanation is the fact that molecular weight determinations based on gel filtration data have given considerably higher values (13, 16) than those obtained from ultracentrifugation analyses (11, 14). However, these discrepancies between the molecular weight determinations may also have other causes (12).

Measurements of nonglobular, flexible molecules like IgA on micrographs is accompanied by certain problems. It is therefore difficult to ascertain the exact point of the bifurcation for the Fab parts and consequently only approximate values can be given for the Fc region. The bending of the molecules, the possible loss of length in the third dimension, the difficulties in making exact measurements with the Brinnel glass, and the subjectiveness of the human eye will also make exact determinations difficult. A slight difference in length or diameter between different molecular populations must therefore be interpreted with caution. We have tried to overcome some of these difficulties when making size determinations by (a) measuring on extraordinarily high magnifications of the molecules, (b) having different persons making measurements, and (c) mixing coded pictures of myeloma dimeric IgA and s-IgA molecules at random before measurements.

According to Brandtzaeg et al. (12) reduction of human s-IgA with a concentration of mercaptoethanol of 0.08 M and subsequent alkylation gave a maximal yield of free, intact S.C. In our hands, only a minor fraction (less than 20%) of the molecules were converted to 7S monomers under these conditions of reduction while the major part was recovered as 11S components with a morphology indistinguishable from that of intact s-IgA. Since the electron micrographs have not permitted a distinction between dimer molecules with or without bound S.C., release of S.C. may have occurred also from the dimer molecules.

The mica technique has been preferable to the conventional staining method

\[1 \text{ Dourmashkin, R. R. Personal communication.} \]
since the former technique permits improved visualization of the molecules which stretch out and spread more evenly on the carbon film. This effect may be due to capillary forces acting when the protein solution is forced up between the mica and the carbon film.

**SUMMARY**

Human colostral IgA and myeloma dimer IgA were purified and examined in the electron microscope using a modified technique of negative staining. Both types of preparation contained double Y-shaped structures of the dimensions: Fab region, 35 × 70 Å, and the sum of the two Fc regions, 40 × 140–155 Å. Colostral IgA as well as myeloma dimer IgA molecules showed a tendency of bending at the point where the Fc regions joined. Secretory component bound to dimer IgA produced no visible alteration of the molecule. Mild reduction and alkylation of colostral IgA yielded single Y-shaped 7S monomers with the dimensions, Fab, 35 × 70 Å, and Fc, 40 × 65–70 Å.

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*Note Added in Proof.*—Dourmashkin and coworkers (personal communication) have recently performed ultrastructural studies on mouse and human myeloma IgA. Their findings on human IgA appear to be in accordance with ours.

**BIBLIOGRAPHY**