PEPTIDE MAPS OF ANTIBODIES AGAINST AN ANTIGEN CONTAINING TWO DIFFERENT DETERMINANT GROUPS.*

Eugene F. Gold, Katherine L. Knight and Felix Haurowitz

(Department of Chemistry, Indiana University, Bloomington, Indiana)

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In order to obtain some insight into the structure of the combining groups of antibodies, we analyzed in earlier experiments of this laboratory (S. Fleischer et al., 1961) the amino acid composition of two azoproteins containing the same protein component but different haptens, namely the acidic azophenylarsonate group (Ars) and the basic azophenyl-N-trimethylammonium group (R_4N). Injection of an azoprotein of this type results in the formation of at least two types of antibodies, one of these combining with the determinant hapten, the other with the protein carrier. The same two haptens were also used by Koshland and Englberger (1963) who isolated the hapten-specific antibodies from the immune serum of single rabbits injected with a mixture of the two azoproteins, and determined their amino acid composition. Peptide maps of the same antibodies from the serum of rabbits injected with either of the two azoproteins were examined by Groff and Haurowitz (1964). The simultaneous injection of the two azoproteins into an animal eliminates differences in the genetic allotypes, but does not exclude the possibility that the distribution of the two antigens in vivo is different and that each of the two antibodies is formed in different tissues. To overcome this difficulty we have injected rabbits with a doubly labelled azoprotein in which diazotized azophenylarsonate and azophenyl-N-tri-

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methylammonium chloride were bound to the same molecules of bovine serum albumin (BSA). We isolated the two hapten-specific antibodies from the immune serum and compared their peptide maps.

Experimental. One half millimole of arsanilic acid (Ars) and the equivalent amount of p-aminophenyl-N-trimethylammonium chloride (R_4N) were diazotized separately in two small beakers in an ice bath as described earlier (Groff and Haurowitz, 1964). 1.0 g of crystalline bovine serum albumin (Nutritional Bioch. Corp.) was dissolved in 25 ml. of 0.15 M phosphate and brought to pH 9 by 0.75 N NaOH. To this solution we added alternately a drop of the two diazotized compounds, maintaining pH between 9.0 and 9.5 by the addition of the required volume of NaOH solution. After exhaustive dialysis against water, the doubly labelled azoprotein solution was made isotonic by the addition of NaCl, and was injected intramuscularly into rabbits with Freunds adjuvant. Separation and isolation of the two hapten-specific antibodies, anti-Ars and anti-R_4N, from each other and from anti-BSA was accomplished by means of an immunosorbent prepared by diazotizing p-aminobenzyl cellulose (Malley and Campbell, 1963) to capacity with nitrous acid, coupling with an equivalent amount of phenol, washing exhaustively, and coupling the product with equivalent amounts of either diazotized arsanilic acid or diazotized p-aminophenyl-N-trimethylammonium chloride. For 100 ml of immune serum a column of 15-20 gram of the immunosorbent was used. After passage of the immune serum, the column was washed with 1% NaCl until only traces of protein were found in the washings. The antibody was eluted by lowering the pH of the NaCl solution to 2.0. The eluate was dialyzed against water, then lyophilyzed. Trypsin digestion of the purified antibody was carried out as described by Groff and Haurowitz (1964).

Electrophoresis of 2.8 or 1.4 mg of the peptide mixture was performed on Whatman 3MM paper between horizontal glass plates in the apparatus of ENSCO (Salt Lake City, Utah) at 18.5 V/cm for 1.5 hours in

Michl's pyridine-acetate buffer at pH 6.4. It was followed by ascending chromatography in butanol-acetic acid-water (4:1:5). The 2.8 mg samples were sprayed with ninhydrin, the 1.4 mg samples with butylhypochlorite and then with a solution containing KI and soluble starch (Mazur et al., 1962).

Results. Whereas the ninhydrin spray reveals the presence of only those peptides which have free primary amino groups, the hypochlorite spray reacts with all peptide bonds. The intensity of its color increases with the chain length of the peptide. Figure 1 and 2 show that the peptide maps of the hapten-specific anti-Ars and anti-R_4N antibodies produced in a single rabbit injected with the doubly labelled antigen are extremely similar, in agreement with results on the same antibodies isolated from rabbits which produced either anti-Ars or anti-R_4N. We find here again only small differences in the peptide maps, indicated in the figures by arrows. The spot a visible in the peptide map of anti-Ars in Fig. 1 and 2, is missing in the map of anti-R_4N. However, the spot b in anti-Ars (Fig. 2) does not appear in Fig. 1.

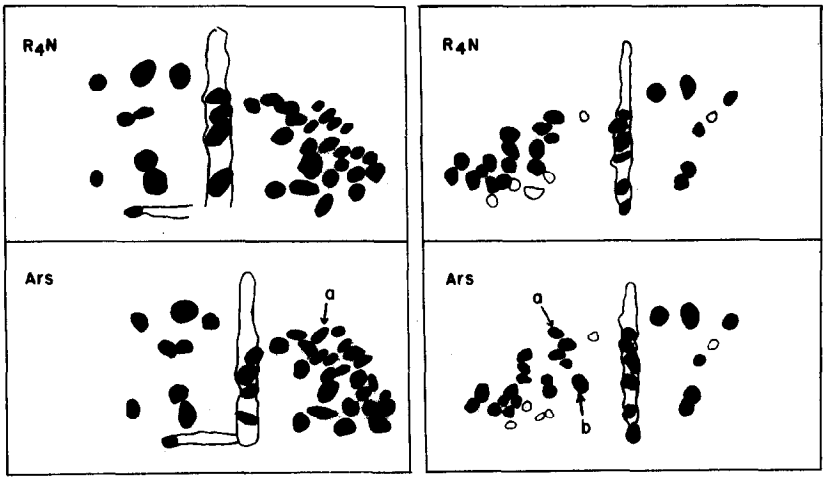

Figure 1 and 2. Peptide maps of Ars-specific and R_4N-specific antibodies isolated from the immune serum of rabbit no. 774 injected with the doubly labelled Ars-R_4N-BSA. In Fig. 1 (left) the paper was exposed to t-butylhypochlorite and then stained with KI and starch. In Fig. 2 (right) the peptide maps were sprayed with ninhydrin. Intensely stained spots are shown in black, faint spots by solid outlines.

Discussion. Slight differences in the peptide maps may be caused by small differences in the experimental conditions during isolation and digestion of the antibodies or during electrophoresis and chromatography. None of these factors can, however, produce identity of the peptide spots in the two antibodies. The identity of most of the peptide spots of the two antibodies indicates clearly that large portions of the two antibody molecules have identical composition and that they differ only in small parts of their molecules. The missing of the spot a in anti-R_4N may indicate one of these differences.

Since we have always found differences in comparing anti-Ars or its fragments I and II with anti-R_4N and its analogous fragments (Groff and Haurowitz, 1964; Gold et al., 1964; Knight et al., 1964), the absence of peptide a in anti-R_4N may be significant. The appearance of spot b after spraying with ninhydrin, but not with butylhypochlorite might indicate the presence of a free amino acid since hypochlorite, in contrast to ninhydrin, would not reveal its presence. Further experiments are necessary to exclude contamination by an extraneous amino acid.

Since the ultracentrifugal analysis of R_4N-BSA, but not of Ars-BSA, revealed the presence of aggregates of high molecular weight, in addition to monomers, differences observed earlier between the peptide maps of anti-R_4N and anti-Ars might have been due to a different organ distribution of the two azoproteins. Differences of this type are eliminated in the work described above since both haptens are coupled to the same protein molecule. On ultracentrifugation the doubly labelled azo-BSA showed only one peak; its sedimentation constant was the same as that of Ars-azo-BSA. Consequently, both haptens must have been carried to the same tissues and cells. Our experiments do not reveal whether the two haptens, after fragmentation of the antigen in the phagocytic cells, remain in the same cell or are transported to differ-

ent cells, and whether the two types of hapten-specific antibodies, anti-Ars and anti-R_4N, are formed in the same or in different cells. It might be possible to answer this question by means of the fluorescent antibody technique.

SUMMARY

Bovine serum albumin doubly labelled by coupling with equivalent amounts of diazotized arsanilic acid (Ars) and p-aminophenyl-N-trimethyl-ammonium chloride (R_4N) was injected into rabbits. Hapten-specific antibodies against the two determinants were separated from each other and from anti-BSA. Peptide maps of anti-Ars and anti-R_4N differ from each other only in one or two spots. Large portions of the two types of antibody molecules seem to be identical. Since both haptens are bound to the same BSA molecules, we assume that they are initially incorporated into the same cells. It is not yet known whether anti-Ars and anti-R_4N are formed in the same cells or whether the haptens, after fragmentation of the antigen, are transferred to different antibody forming cells.

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