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Preparation and properties of an artificial antigen immunologically related to tobacco mosaic virus

The first artificial antigen which showed an immunological relationship to a natural antigen was prepared by GOEBEL[1,2]. He found cellobiuronic acid to be the determinant group of the capsular polysaccharide antigen of pneumococcus type III and obtained the artificial antigen by coupling diazotized *p*-aminobenzyl cellobiuronide to protein. The antisera which he prepared strongly precipitated type III polysaccharide and agglutinated type III pneumococci. More recent investigations on certain pathogenic Gram-negative bacteria[3] led to the synthesis of an artificial antigen which contained α-phenyl-colito-pyranoside linked to bovine serum albumin by a diazo group[4]. Various bacterial polysaccharides containing terminal colitose cross-reacted with antisera to the artificial colitose antigens, whereas some of these antisera even agglutinated *Escherichia coli* O III suspensions.

GOEBEL demonstrated that immunization with artificial antigens gives effective immunological protection against otherwise fatal infections. From this point of view it is of considerable interest to find methods for the preparation of artificial antigens which give immunological protection against natural protein antigens such as simple viruses with a protein coat. As far as we know these coat proteins are of the globular type. The search for serologically active sites of globular proteins has not yet been as successful as corresponding investigations carried out with polysaccharide antigens. This failure is partly due to the fact that the determination of amino acid sequences has become possible only during the past years, partly to the rather complex structure of globular proteins.

It is now generally believed that the serological specificity of globular proteins is dependent not only on distinct regions of the primary structure, but also on their spatial arrangement[5]. This has been proved for tobacco mosaic virus by renaturation experiments with chemically altered tobacco-mosaic-virus protein and the study of its serological behaviour[6]. Though the chemical structure of the complete combining site of tobacco mosaic virus is still unknown, it is fairly certain that the C-terminal sequence of the polypeptide chains takes part in it. Experiments[7] based on the inhibition of the specific precipitation of tobacco mosaic virus with the corresponding antibodies using various peptides of tobacco mosaic virus as haptens showed that only a few peptides accounting for at most 20 % of the polypeptide chain can diminish quantitative precipitation. The C-terminal peptides[8] at Position 142–158, Position 151–158, and Position 153–158 all had a similar inhibition capacity. Thus only the terminal hexapeptide (or even a shorter sequence) seems to be involved in the reaction. This was to be expected from the experiments of HARRIS AND KNIGHT[9] who showed that the C-terminal groups are accessible to enzymes and are therefore located on the virus surface.

For further investigations on the determinant role of the C-terminal sequence of tobacco mosaic virus an artificial antigen containing the C-terminal hexapeptide linked to a carrier protein has been prepared. Although the C-terminal hexapeptide does not represent the complete combining site of tobacco mosaic virus, a serological cross reaction could be expected.

Preparation of the hexapeptide antigen. Since the antigenicity of an artificial antigen is dependent on the antigenicity of the carrier protein, purified bovine serum

albumin was cross-linked according to the method of SCHICK AND SINGER[10]. For the preparation of the hexapeptide antigen the peptide Thr–Ser–Gly–Pro–Ala–Thr was prepared by enzymic digestion of tobacco-mosaic-virus protein and N-acylated by treatment with *p*-nitrobenzoylchloride at pH 8.8, 40° and converted to the *p*-aminobenzoyl derivative by catalytic hydrogenation. The diazotized sample was added to a solution of cross-linked bovine serum albumin in 0.2 M sodium acetate (pH 9.0) at 0° and kept at 4° overnight. The orange-coloured solution of the azoprotein was dialysed against 10^{-3} N NaOH and water and afterwards lyophilized. The azoprotein contained 1 mg of bound hexapeptide per 27 mg of cross-linked bovine serum albumin estimated by extinction measurements; intact tobacco mosaic virus contains 1 mg hexapeptide per 35 mg virus.

Serological tests. Rabbits were injected with the hexapeptide antigen, with cross-linked bovine serum albumin and with tobacco mosaic virus strain vulgare. All three antigens did not react with sera isolated before immunization. All sera isolated after immunization contained precipitating antibodies against the homologous antigens. All three antigens gave precipitation with the antiserum to the hexapeptide antigen. As can be seen from Fig. 1, the highest amount of precipitate was obtained with the homologous hexapeptide antigen, whereas carrier protein and tobacco mosaic virus gave smaller precipitates. The tobacco mosaic virus–antibody complex is less soluble in the zone of antigen excess than the other precipitates. Therefore the equivalent zone is broader.

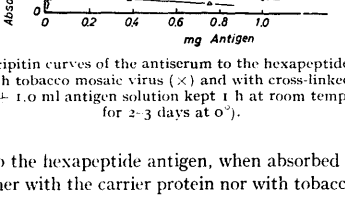

Fig. 1. Quantitative precipitin curves of the antiserum to the hexapeptide antigen with the hexapeptide antigen (○), with tobacco mosaic virus (×) and with cross-linked bovine serum albumin (△). (0.1 ml antiserum + 1.0 ml antigen solution kept 1 h at room temperature and then stored for 2–3 days at 0°).

The antiserum to the hexapeptide antigen, when absorbed with the homologous antigen, reacted neither with the carrier protein nor with tobacco mosaic virus. After absorption with tobacco mosaic virus the serum still reacted with the carrier protein and the hexapeptide antigen and after absorption with the carrier protein it still reacted with tobacco mosaic virus and the hexapeptide antigen.

Reciprocal cross reaction could be demonstrated between the hexapeptide antigen and the carrier protein and their antisera. Using antisera with comparable titers the quantitative precipitin curves of the homologous and the heterologous reactions were approximately the same. On the other hand the hexapeptide antigen does not give any precipitations with anti-tobacco mosaic virus serum and scarcely inhibits (10–15 %) the tobacco mosaic virus/anti-tobacco mosaic virus reaction when applied in 50-fold excess calculated on the hexapeptide content.

The fact that the cross reaction between tobacco mosaic virus and the hexapeptide antigen is of the areciprocal type can be understood in terms of steric inter-

actions. If the hexapeptide is in a relatively exposed position on the tobacco-mosaic-virus surface, then the antibody against the hexapeptide antigen would be expected to react with tobacco mosaic virus. However, if the total combining site on tobacco mosaic virus is considerably more complex than just the hexapeptide, the specific antibody against this site would not necessarily react with the simple hexapeptide antigen.

This idea is further supported by inhibition experiments under comparable conditions. The hexapeptide inhibits the tobacco mosaic virus/anti-tobacco mosaic virus reaction by about 20 % under the most favorable conditions[7] whereas it inhibits the reaction between tobacco mosaic virus and the antiserum to the hexapeptide antigen by up to 75–85 %. This effect can be understood if the combining site of tobacco mosaic virus is considerably more complex than just the hexapeptide.

The antibodies against the hexapeptide antigen are directed specifically against the sequence Thr–Ser–Gly–Pro–Ala–Thr. Cross-reaction with tobacco mosaic virus strain Dahlemense does not occur, although the sequence of its C-terminal hexapeptide Thr–Ser–Ala–Pro–Ala–Ser[11] is very similar to that of tobacco mosaic virus strain vulgare.

To demonstrate neutralising activity of the antiserum to the hexapeptide antigen solutions of tobacco mosaic virus up to 10 μg/ml were mixed with an excess of the serum and applied to tobacco plants (Xanthi). No lesions could be observed. Thus the serum against the hexapeptide antigen is as effective in inhibiting infection with the virus as is the homologous antiserum.

In summary peptides containing the C-terminal sequence of tobacco mosaic virus diminish the precipitation of this virus with its antiserum. A synthetic antigen with the C-terminal hexapeptide of the polypeptide chain of tobacco mosaic virus as determinant group produces an antiserum which precipitates and neutralizes tobacco mosaic virus. The antiserum does not react with the strain Dahlemense which has a similar C-terminal sequence. The artificial antigen does not precipitate with anti-tobacco mosaic virus serum. Inhibition experiments and the lack of reciprocal cross reaction provide evidence that the combining site of tobacco mosaic virus for the antibody is larger than the hexapeptide.

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