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PREPARATION AND EVALUATION OF ANTI-SPECIES AND ANTIVIRAL PEROXIDASE CONJUGATES

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Summary. — Several antispecies peroxidase conjugates from human and rabbit immunoglobulins G (IgG) and a conjugate from IgG fraction of hyperimmune rabbit serum against the membranes of herpes simplex virus type 1 (HSV-1) infected cells have been prepared and characterized. The conjugates when tested in enzyme immunoassay for detection of antiherpetic antibodies in human and hyperimmune rabbit sera, as well as for detection of HSV-1 antigen in infected Vero cells appeared active and highly specific. Comparison with the peroxidase conjugates from antiherpetic IgGs prepared by means of ion- exchange and affinity chromatography has shown similar activities.

Key words: peroxidase conjugate; class G immunoglobulins (IgG), affinity chromatography; protein A Sepharose 4B; enzyme immunoassay; herpes simplex virus type 1

Introduction

Detection of viral antibodies in enzyme-linked immunosorbent assay (ELISA), peroxidase conjugates included, requires highly active and specific conjugates based on immunoglobulins of such animal or human species whose sera are being analysed. Furthermore, of importance is also the detection of viral antigens, for which peroxidase conjugates prepared on the basis of antiviral IgG antibody fractions are used.

At present, a number of techniques has been developed for preparation of homogeneous IgG-fractions not contaminated with other class immunoglobulins (Reif, 1969; Baumstark, Laffin, 1964; Kronvall, Frommel, 1970) and for production of conjugates from them (Kurstak and Kurstak, 1974; Nakane and Kawaoi, 1974; Wilson and Nakane, 1978). We report the preparation of a series of peroxidase labelled conjugates from human and rabbit IgG and from hyperimmune rabbit IgG rised against HSV-1 infected cell membranes. We aimed at use of IgG isolated by affinity chromatography on protein A-Sepharose 4B (a method which seems to have advantages over techniques such as ion-exchange chromatography on DEAE-cellulose or on DEAE-Sephadex A-50). We compared these methods in terms of production

of conjugates with optimal antiviral potency (using antiherpetic IgGs as example) for detection of HSV-1 antigen in ELISA and finally, to estimate the activity and specificity of the peroxidase conjugates employing a domestic enzyme.

Materials and Methods

Sera. Swine serum against human and rabbit IgG were commercial sera obtained from the Institute of Sera and Vaccines (Sevac, Prague, ČSSR). Hyperimmune serum to HSV-1 and against HSV-infected cell membranes have been prepared in rabbits by three consecutive intramuscular immunizations at three-week intervals using equal volumes of complete Freund's adjuvant. The serum titres in immunodiffusion test in the agar were 32 to 64.

Horse radish peroxidase with RZ-3 was a gift of NPO Biokhimreaktiv (Olaine, Latvian Soviet Socialist republic). Protein A-Sepharose 4B (Farmacia Fine Chemicals) and Servacel DEAE 22 (Serva) were used. The titrations were carried out in 96-well polystyrene plates (All-Union Research Institute of Experimental Medical Equipment).

The IgG fractions were isolated by modified affinity chromatography on protein A-Sepharose 4B (Hjelm *et al.*, 1972). Briefly, 2 ml of serum was passed through a minicolumn (5×1.5 cm), washed with several volumes of 0.01 mol/l Na phosphate buffer pH 7.2 and the IgGs were eluted by 0.58 % CH_3COOH solution in 0.85 % NaCl. The eluted fractions were passed through continuous flow cell Uvicord (LKB, Sweden) and the yield of the material was detected by monitoring of the optical density at 280 nm. Peak fractions were combined, the material contained in them was neutralized with 0.2 mol/l Tris-HCl buffer and centrifuged at low speed ($2.5 \times 10^3 \times g$ for 10 min) to remove protein aggregates. The material was then dialyzed overnight against Na-carbonate buffer pH 9.2 at 4 °C.

The peroxidase conjugate was prepared by the modified technique of Tijssen and Kurstak (1982). The homogeneity of peroxidase conjugates was assessed by gel-filtration. The material was analysed on Sephadex G-200 column (30×0.6 cm) equilibrated with 0.01 mol/l Na-phosphate buffer pH 7.2 at elution rate of 40 ml/hr. The volume of collected fractions was 2 ml. Protein content in the eluates was determined by measuring of absorbancy at 254 and 403 nm.

Immunodiffusion in agar. Agarose solution (1 %) in 0.025 mol/l Tris-HCl buffer pH 8.0 with 0.2 % (w/v) of Triton X-100 was used. The 10 µl samples were introduced into the wells and immunodiffusion proceeded for 24 hr at room temperature. Precipitates were photographed without pre-treatment.

Enzyme immunoassay was carried out according to a conventional method (Voller and Bidwell, 1975) in 50 µl volume. The direct ELISA has been performed. HSV-1 (strain VR-3)-infected Vero cells were used for adsorbtion. Each serum was assayed against the adsorbed virus and control antigens. Uninfected Vero cells in the same concentration (10^4 cells per well) were used as controls.

For detection of HSV-1 antigen, the microtitre plate wells were adsorbed in even rows with rabbit antiherpetic IgG-antibodies and in odd rows with the IgGs isolated from normal rabbit serum. Virus-containing cell suspension was pretreated as described (Land *et al.* 1984) at a concentration of 10—50 ng per 50 µl and was tested against antiviral and normal immunoglobulins.

Results

Fig. 1 shows the results of a typical experiment for the preparation of swine serum IgG fraction against human IgG by affinity chromatography on protein A-Sepharose 4B. This technique is based on the high affinity of IgG Fc fragments to active centres of *Staphylococcus aureus* protein A (Kronvall and Frommel, 1970; Sjoqvist *et al.*, 1976). First peak (I) represents the serum material which failed to bind to the column. The application of elution buffer consisting of 0.58 % acetic acid (pH 3.0) onto the column

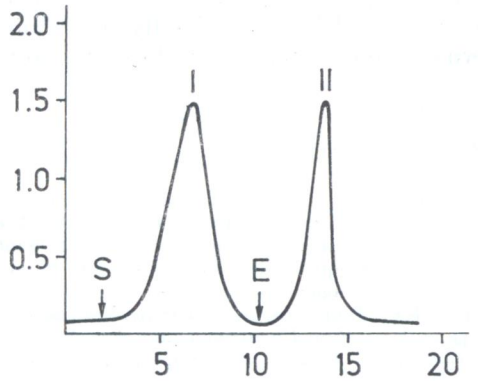

Fig. 1.

Preparation of IgG by affinity chromatography on protein A-Sepharose 4B
2 ml of serum sample has been passed through the column (5 × 1.5 cm), washed with several volumes of 0.01 mol/l Na-phosphate buffer pH 7.2; the IgG was eluted with 0.85 % NaCl in 0.58 % CH_3COOH solution. Fractions were passed through Uvicord flow cell and the yield was detected by monitoring of the optical density at 254 nm. S = serum; E = elution. Peak I — material not bound by the sorbent, peak II — IgG preparation. Abscissa: fraction numbers. Ordinate: E_{254} nm.

resulted in release of IgG fraction as evident from optical density at 254 nm (peak II).

Immunodiffusion in agar demonstrated that the prepared IgG fractions were not contaminated by immunoglobulins of other classes. Precipitation line was formed only between the wells containing anti-IgG and IgG, whereas the tested material failed to react with IgM and IgA.

The conjugates were prepared according to Tijseen and Kurstak (1982) using slightly modified ratios of enzyme and immunoglobulin in the conjugation mixture. The conjugates were tested for the presence of unconjugated IgG molecules on Sephadex G-200. It has been shown that the conjugate migrated as one homogeneous peak (Fig. 2) and no free immunoglobulin molecules have been revealed.

For the evaluation of the quality of conjugates the molar ratio of IgG to peroxidase has been calculated. It has been found that in conjugates with optimal properties the optical density ratio at 403 and 278 nm did not fall

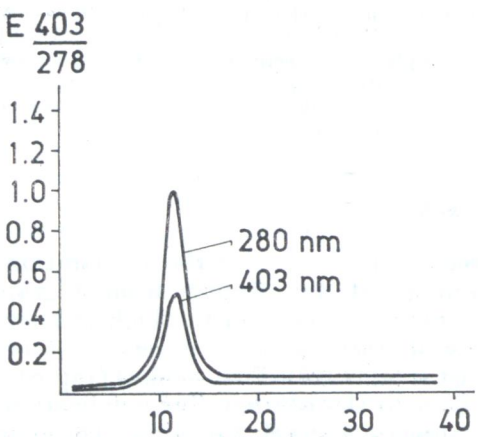

Fig. 2.

Determination of peroxidase conjugate homogeneity by gel-filtration technique
The material was applied on Sephadex G-200 column (30 × 0.6 cm) equilibrated with 0.01 mol/l Na-phosphate buffer pH 7.2 at flow rate of 40 ml/hr. The fraction volume was 2 ml. The protein content was measured in the eluates by absorption at 278 and 403 nnm. Abscissa: fraction numbers. Ordinate: E ratio 403 to 278 nm.

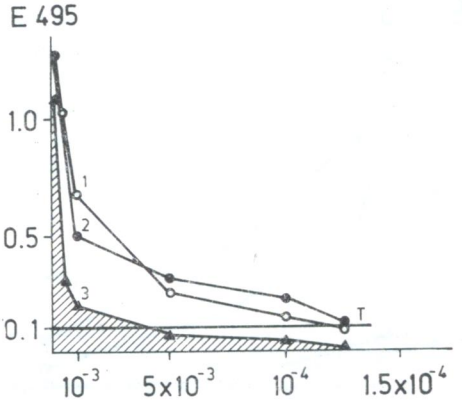

Fig. 3.
Determination of antihuman peroxidase conjugate titre (batches 1 and 3) in ELISA
ELISA was performed according to the classic method of Voller and Bidwell (1975) in 50 ml volume; the direct test has been used. HSV-1 infected Vero cells were absorbed to the wells as antigen. Each serum with a known titre was tested with the absorbed virus and control antigens. The control value ("threshold" value "T") was computed from values obtained with human serum lacking antibodies to HSV-1 (titre lower than 90). 1 — human serum (dilution 1 : 7290) — antihuman conjugate (batch 1), 2 — human serum (dilution 1 : 7290) — antihuman conjugate (batch 3), 3 — background values. Here and in Figs 4 and 5 the abscissa: conjugate dilutions.

below 0.4 (Nakane, 1975). In our case this ratio was 0.5 (Fig. 2). Thus, based on accepted biochemical evaluation of conjugates it may be concluded that homogeneous preparations can be produced without additional purification.

Immunological specificity and activity of prepared antihuman and antirabbit conjugates has been assessed in ELISA designed for detection of antiherpetic IgG antibodies in human sera and hyperimmune rabbit sera. For determination of working dilutions of the conjugates to different immunosorbents standard sera at dilutions corresponding to their titre have been examined. To establish the control level ("threshold value"), human serum lacking antibodies against HSV-1 (titre lower than 1 : 90), as well as normal rabbit sera at the same dilutions as the antiviral ones, have been tested in parallel against viral and control antigens. The antihuman and antirabbit conjugate batches at different dilutions served as detector antibodies.

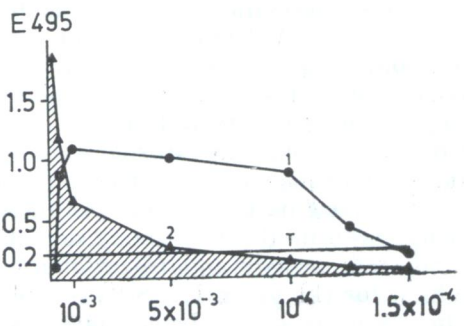

Fig. 4.
Determination of antirabbit peroxidase conjugate titre in ELISA
Control values have been computed on the basis of normal rabbit sera in the same dilutions as the immune ones. 1 — hyperimmune anti-HSV-1 rabbit serum (dilution 1 : 81,000), 2 — background values. Abscissa: conjugate dilutions.

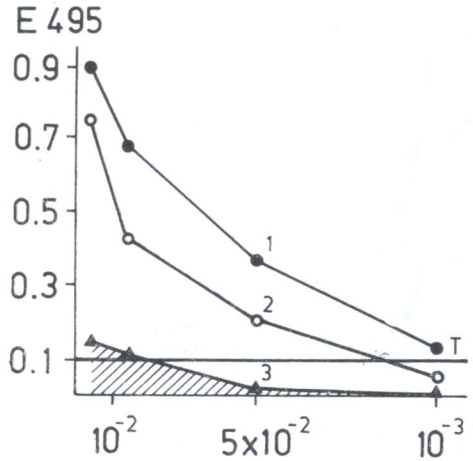

Fig. 5.

Determination of antiherpetic peroxidase conjugate titre

To detect the HSV-1 antigen both rabbit antiherpetic IgG fraction and IgG isolated from normal rabbit serum have been adsorbed to the wells of polystyrene plates. Virus-containing suspension was pretreated as described by Land *et. al.* (1984) and tested at concentrations of 10—50 ng per 50 μl with immune and nonimmune immunoglobulins. "Threshold" values (T) were determined as described in Results. 1 — anti-HSV-1 IgG (protein A) conjugate, 2 — anti-HSV-1 IgG (DEAE) conjugate, 3 — — background values. Abscissa: conjugate dilutions.

The results of these experiments on example of two batches of antihuman peroxidase conjugate and one antirabbit conjugate are shown in Figs. 3 and 4. The difference between the optical densities of viral and control antigens at 495 nm (background values) has been computed; values below the control level have been considered as negative and have not been taken into account for titre determination. Thus, the conjugate titre was defined as numerical value of its limiting dilution, provided that it was higher than the threshold level evaluated for the given ELISA. It can be seen from the figures that the titre of antirabbit conjugate was 1 : 12,000 and that of antihuman conjugates was 1 : 10,000.

Further we have estimated the activity and specificity of peroxidase conjugates prepared from antiherpetic IgG class antibodies obtained by means of two methods — affinity chromatography on protein A-Sepharose 4B and by ion-exchange chromatography on DEAE-cellulose. The conjugates were tested in ELISA for HSV-1 antigen with the help of the direct double antibody method. To determinate the working dilutions of conjugates HSV-1 infected cells treated as described by Land *et al.* (1984) and containing about 40 ng viral protein have been tested on polystyrene immunosorbents with HSV-1 antimembrane serum and normal rabbit serum.

For control of ELISA we used: 1) "normal" rabbit immunoglobulins, 2) "quenching" test by means of preliminary incubation of the virus-containing suspension with antiherpetic serum, 3) conjugate control using buffer solution instead of virus-containing material in the test and, finally, 4) uninfected cells treated in the same manner as the infected ones.

The analysed batch of anti-HSV-peroxidase conjugates at different dilutions has been used for antibody detection. Determination of control values allowed preliminary standardization and establishment of the optical density threshold for the given ELISA system. Threshold value was defined as the arithmetic mean of the background values (quenching test, conjugate con-

trol, etc.) plus double standard deviation from this value. The results were considered positive if the difference between optical densities of the tested material at 495 nm and control samples reacting with virus-specific immunoglobulins was higher than the threshold value.

The results of a typical experiment are shown in Fig. 5. The conjugate titres have been determined as described above and equaled 1 : 1000 for anti-HSV-1 IgG (protein A) and 1 : 500 for anti-HSV-1 IgG (DEAE).

Discussion

Several IgG samples have been prepared by affinity chromatography on protein A-Sepharose 4B with the aim to use them for peroxidase conjugate production. The advantage of this technique over conventional method of ion-exchange chromatography on DEAE-cellulose is that it allows to eliminate the stage of common immunoglobulin pool production via precipitation of hyperimmune serum by ammonium sulphate. It has been shown that the preparations tested were not contaminated by other immunoglobulin classes and were functionally active protein molecules.

As it has been mentioned above, several methods can be applied for the preparation of peroxidase conjugates on IgG-antibody basis. Wicker and Avrameas (1969) have proposed a method of antibody conjugation with peroxidase with the bifunctional reagent — glutaraldehyde. However, this method tends to leave free unconjugated molecules of IgG and of the enzyme, so that additional gel-filtration on Sephadex G-200 (Kurstak and Kurstak, 1974) or affinity chromatography on protein A-Sepharose 4B column followed by rechromatography on Concanavaline A-Sepharose (Boorsma and Steefkerk, 1978) are needed to eliminate them. Nakane and Kawaoi (1974) have found that during the application of glutaraldehyde only 2 to 4 % of the added peroxidase bound to IgG. Therefore, they have proposed another conjugation method based on activation of the enzyme by sodium periodate which does not interfere with enzymatic activity and produces active aldehyde groups capable of interaction with the immunoglobulin amino groups. This method was widely adopted in experimental research and later modified by Tijssen and Kurstak (1982). It has been used in this paper; our modification was in changing the molar ratios of IgG to the enzyme added into the conjugation mixture.

As mentioned, the presence of free immunoglobulin and enzyme molecules in the conjugate inhibits its specific action in the immunoenzyme assay. The unliked peroxidase molecules we eliminated by precipitation of the conjugate with ammonium sulphate. As far as the probable presence of free IgG molecules concerned, the proposed modification allowed us to expect that their number was insignificant. We have demonstrated that the prepared conjugates migrated at gel-filtration on Sephadex G-200 as one homogeneous peak indicating the absence of unconjugated IgG molecules or, at least, that their number was insignificant and unidentifiable. Moreover, the high specificity of conjugates as determined in ELISA confirmed this suggestion. Thus,

in the limits of the adopted conjugate assessment it may be concluded that they can be used without additional purification.

As it has been mentioned, immunologic specificity and activity of the prepared anti-species conjugates have been assessed in appropriate ELISA system. It has been shown that the titres of antirabbit conjugate are 1 : 12,000 and of antihuman one they equal 1 : 10,000. Furthermore, such a high activity of the produced conjugates was associated with high specificity of their action (see background values and "threshold level" in the Figs. 3 and 4). It is noteworthy that low dilutions of the conjugate (1 : 100, 1 : 500) exhibited marked nonspecific reactions which ruled out the possibility of their use.

Further research was devoted to the assessment of the activity and specificity of peroxidase conjugates prepared on the basis of antiherpetic IgG--antibodies for detection of HSV-1 antigen in ELISA. As it has been mentioned, IgG fractions were produced by two different methods: affinity chromatography on protein A-Sepharose 4B and by ion-exchange chromatography on DEAE-cellulose. The need for comparative analysis of the activity of conjugates with isolated IgG fractions came from the available evidence that immunoglobulins of subclass 3 (IgG 3) whose Fc receptors have no affinity for the active centres of protein A possess the highest antiviral activity (Hjelm *et al.*, 1972). Our findings have shown that the conjugates tested in the given ELISA had a comparable activity. At the likelihood of 5 % contribution of IgG 3 to the total immunoglobulin pool (Sundqvist *et al.*, 1984) it could exert a significant influence on the conjugate activity in detection of viral antigen.

References

Baumstark, J. S., and Laffin, R. J. (1964): A preparative method of the separation of 7S gamma globulin from human serum. *Arch. Biol. Biophys.* **108**, 514—522.

Boorsma, D. M., and Streefkerk, J. G. (1978): Improved method of separation of peroxidase conjugates, pp. 225—235. In W. Knapp, K. Holobar, G. Wick (Eds): Proc of the 6-th *Intern. Conf. of the Immunofluor. and Related staining Techniques*, Vienna.

Hjelm, H. (1975): Isolation of IgG3 from normal human sera and from patients with multiple myelomatosis by using protein A-Sepharose 4B. *Scand. J. Immunol.* **4**, 633—640.

Hjelm, H., Hjelm, K., and Sjoqvist, J. (1972): Protein A from *Staphylococcus aureus*. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. *FEBS Letters* **28**, 73—76.

Kronvall, G., and Frommel, D. (1970): A reactivity of human immunoglobulin G fragments. *Immunochemistry* **7**, 124—127.

Kurstak, E., and Kurstak, C. (1974): Application of the immunoperoxidase technique in virology and cancer virology, light and electron microscopy, pp. 3—33. In E. Kurstak, R. Morisset (Eds): *Viral Immunodiagnosis*. Acad. Press, New York, San Francisco, London.

Land, S.-A., Skurrie, J., and Gilbert, G. (1984): Rapid diagnosis of herpes simplex virus infections by enzyme-linked immunosorbent assay. *J. clin. Microbiol.* **19**, 865—869.

Nakane, P. K., and Kawaoi, A. (1974): Peroxidase-labelled antibody: a new method of conjugation. *J. Histo hem. Cytochem.* **22**, 1084—1091.

Nakane, P. K. (1975): Recent progress in the peroxidase-labelled antibody method. *Ann. N.Y Acad. Sci.* **254**, 203—211.

Reif, A. (1969): Batch preparation of rabbit G-globulin with DEAE-cellulose. *Immunochemistry* **6**, 723—731.

Sjoqvist, J., Forsgren, A., and Gustafson, G. (1976): Interaction between gamma globulins and protein A from *Staphylococcus aureus*. *Nobel. Symp.* **3**, 341—348.

Sundqvist, V.-A., Linde, A., and Wahren, B. (1984): Virus specific immunoglobulin G subclasses in herpes simplex and varicella zoster virus infections. *J. clin. Microbiol.* **20**, 94—98.

Tijsen, P., Su, D.-M., and Kurstak, E. (1982): Rapid and sensitive heterologous enzyme immunoassays for Densonucleosis virus (Parvoviridae). *Arch. Virol.* **74**, 277—291.

Voller, A., and Bidwell, D. (1975): A simple method for detection of antibodies to rubella. *Brit. J. exp. Path.* **56**, 338—339.

Wicker, R., and Avrameas, S. (1969): Localization of virus antigens by enzyme-labelled antibodies. *J. gen. Virol.* **4**, 465—471.

Wilson, M., and Nakane, P. (1978): Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies, pp. 215—224. In W. Knapp, K. Holobar, G. Wick (Eds): *Proc. of the 6-th Intern. Conf. of the Immunofluor. and Related Staining Techniques*, Vienna.