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Immunogenicity of Heterologous Fc and Fab Immunoglobulin Fragments in Rabbits, Guinea-Pigs and Rats

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Summary. Rabbits, guinea-pigs and rats were immunized with various heterologous 7S and 19S immunoglobulins from each other and man, and the antisera obtained were studied by immunoelectrophoresis.

Rabbits produced antibodies against the Fc and the Fab fragments of the immunoglobulin injected, while guinea-pigs and rats only produced anti-Fc antibodies.

The fact that guinea-pigs and rats only respond to the specific determinants of each class of immunoglobulin provides a simple method for the preparation of class-specific antisera.

INTRODUCTION

One of the problems frequently encountered in all immunological laboratories is the preparation of potent antisera specific for different immunoglobulins. Most of the antisera currently employed are prepared either in rabbits, when the preparation is done in a laboratory, or in horses, goats and sheep, on a commercial basis. These species are good antibody producers and a substantial yield of serum can be obtained from each animal.

When one of these animals is injected with a heterologous γ-globulin, the antibody produced can be directed against antigenic determinants present in the various polypeptide chains of the molecule. In order to prepare specific antisera revealing only one type of immunoglobulin, it is necessary to absorb the antiserum with all the other immunoglobulins sharing common antigenic determinants.

We have recently observed that the immunogenicity of heterologous immunoglobulins in rats and guinea-pigs, is predominantly due to determinants present in the Fc fragment. This fact, and the development of immunization schedules resulting in fast and strong antibody production in these species, have been used to advantage in the preparation of potent antisera which, in most cases, are specific for the immunoglobulin used for immunization.

MATERIALS AND METHODS

Immunoglobulins

IgG globulins from man, guinea-pig and rat were obtained from normal serum, by DEAE-cellulose chromatography with 0·005 M phosphate buffer, pH 8·0. Guinea-pig γ_1-globulin was obtained by DEAE-cellulose chromatography of purified anti-dinitrophenyl antibody, as previously described (Oettgen, Binaghi and Benacerraf, 1965). Human IgM was isolated from sera of cirrhotic patients. After dialysis of the serum against

distilled water, the precipitate formed was collected, washed, dissolved in Tris buffer (0·1 M Tris, 1 M NaCl, pH 8·0) and applied to a Sephadex G-200 column equilibrated with the same buffer. The first fraction eluted was used to inoculate the animals. Guinea-pig IgM was obtained from normal guinea-pig serum by the same method used for human IgM. Rat IgM was obtained by Sephadex G-200 fractionation of purified rat anti-dinitrophenyl antibodies (Binaghi, Benacerraf, Bloch and Kourilsky 1964).

Papain digestion of immunoglobulins

The method described by Porter (1959) was employed. Ten milligrams of protein were dissolved in 1 ml phosphate buffer 0·1 M, pH 7·5, made 0·003 M in EDTA and 0·01 M in cysteine. Crystalline papain, 0·15 mg (Koch-Light Laboratories Ltd, England), was added, and digestion was carried out for 3 hours at 37°. The reaction was stopped by the addition of 0·2 ml of 0·1 M Na iodoacetate, pH 7·5, and the digest was dialysed overnight against saline.

Preparation of $(Fab)_2$ fragment of immunoglobulin

The method described by Nisonoff, Wissler, Lipman and Woernley (1960) was employed. Ten milligrams of protein dissolved in 1 ml acetic buffer 0·1 M, pH 4·5, were digested with 0·2 mg crystalline pepsin (Koch-Light Laboratories) for 8 hours at 37°. After digestion, the pH was adjusted to 8·0 and the $(Fab)_2$ fragment was precipitated at 18 per cent Na_2SO_4, dissolved in 1 ml water and dialysed against buffered saline, pH 7·5.

Immunization of guinea-pigs

Random bred guinea-pigs of both sexes, weighing 300–500 g were used. The various immunoglobulins were dissolved in saline and emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.). Each animal received 0·5 ml of the emulsion intramuscularly into each hind leg. The total quantity of protein inoculated in each animal was between 0·1 and 0·5 mg. One week later, the animals were re-inoculated with the same dosage, 0·4 ml into each hind leg and 0·1 ml intradermally in two sites on the back. Three to 4 weeks after the second injection, serum was collected by carotid section. Seven to 10 ml of serum can be obtained from each guinea-pig. This method of immunization determines a strong antibody production, and most of the sera contained 2–5 mg/ml of antibody (Binaghi, 1966).

Immunization of rabbits

Adult rabbits were inoculated in the same way and with the same dosage as described for guinea-pigs. In some cases, the animals were boosted 4–6 weeks after the first injection, and bled 10 days later.

Immunization of rats

Wistar rats, weighing 250–400 g were used. Female animals were preferred, as they produce more antibody (Oettgen, Binaghi and Benacerraf, 1966). The animals were inoculated in the four footpads with a total dosage of 0·2–0·5 mg protein emulsified in 1 ml of complete Freund's adjuvant. Ten to 15 days later, the animals were bled from the retro-orbital plexus (Halpern and Pacaud, 1951), and the sera were tested for antibodies. At that time, if necessary, a booster injection with the same dosage of antigen emulsified in complete Freund's adjuvant was given in the footpads. One week later, serum was

collected by cardiac puncture. Usually, 5–7 ml of serum can be obtained from each animal.

Immunoelectrophoretic analysis

Immunoelectrophoresis was carried out by the method of Scheidegger (1955), using L.K.B. equipment (Stockholm, Sweden); 1·25 per cent agar in veronal buffer, 0·025 M, pH 8·2, was used. The electrode vessels were filled with veronal buffer, 0·05 M, pH 8·2.

RESULTS

Rabbits, guinea-pigs and rats were immunized with various 7S and 19S heterologous immunoglobulins. At least six animals were immunized with each antigen and the sera were individually studied by immunoelectrophoresis.

RABBIT ANTISERA

Rabbits immunized with immunoglobulins from man, guinea-pig or rat produced antibodies directed against both Fc and Fab fragments of the immunoglobulin molecule. This is clearly evident in the immunoelectrophoretic analysis of the sera against the papain digests of human, guinea-pig and rat IgG, shown in Fig. 1(a), (b) and (d). It can be seen that two arcs of precipitation are formed in all cases, the faster one corresponding to the Fc fragment and the slower one to the Fab fragment. The existence of antibodies against Fc and Fab fragments is also shown in Fig. 2(a), where guinea-pig serum has been developed with two rabbit antisera obtained by immunization with guinea-pig γ_1- and γ_2-globulin, respectively. It can be observed that each antiserum reacts with the specific determinants of the immunoglobulin used as antigen, but also possesses antibodies directed against the common determinants of γ_1 and γ_2, namely their Fab fragments.

Immunization of rabbits with purified preparations of human, rat or guinea-pig 19S immunoglobulins resulted in the production of antibodies which reacted not only with 19S but also with 7S immunoglobulins. An example is presented in Fig. 2(c). In this immunoelectrophoresis, a rabbit antiserum against human macroglobulin reveals three components: IgM, IgG and another serum component in the anodic region. The latter one is probably an impurity in the preparation used for immunization. The arc corresponding to IgG is due to antibodies directed against the Fab fragment, as shown in the same immunoelectrophoresis when the antiserum reacts with a papain digest of human IgG.

Some rabbit antisera against human IgG and guinea-pig γ_1- or γ_2-globulins were quantitatively analysed by the precipitin technique, using either whole γ-globulin or the corresponding $(Fab)_2$ fragment as antigen. It was found that more antibody was present against Fab than against Fc fragment.

It can be concluded from these results that both Fc and Fab fragments of the heterologous immunoglobulins employed in this study possess determinants which are immunogenic in rabbits.

GUINEA-PIG AND RAT ANTISERA

Guinea-pigs and rats immunized with various heterologous normal immunoglobulins produced antibodies directed mainly, if not only, against the Fc fragment of the immunoglobulin employed for immunization. This is shown by the formation of only one arc of precipitation in immunoelectrophoresis of the papain digest of the immunoglobulin.

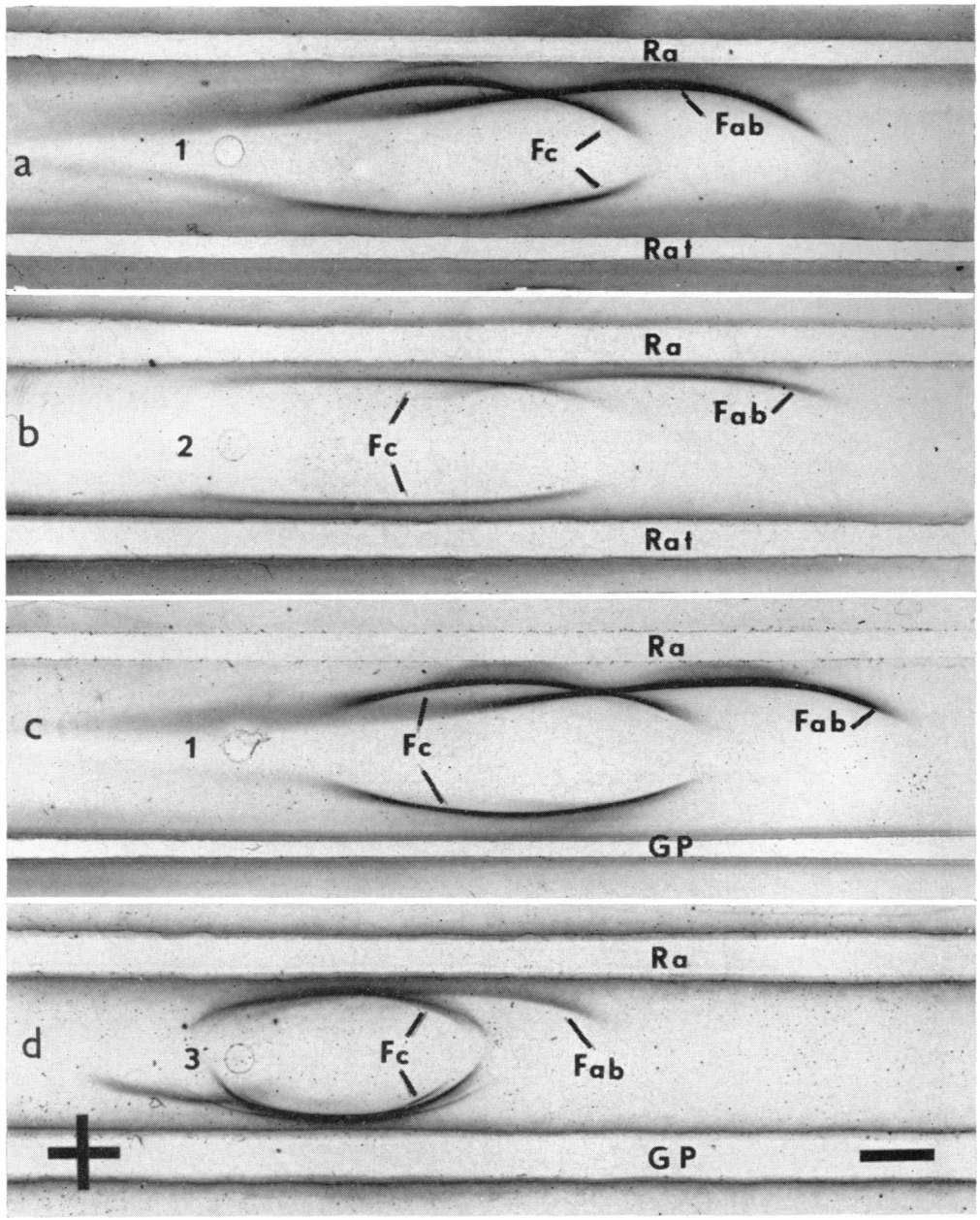

FIG. 1. Immunoelectrophoresis of papain digests of human (1), guinea-pig (2) and rat (3) IgG, developed with antisera obtained in rabbits (Ra), guinea-pigs (GP) and rats by immunization with the corresponding undigested IgG. The guinea-pig anti-rat IgG employed in (d) detects a serum component in the anodic region whose identity is not clear at present.

Rats immunized with human IgG or guinea-pig γ_1- or γ_2-globulins did not produce any antibody against Fab fragment detectable with the technique employed. Fig. 1(a)

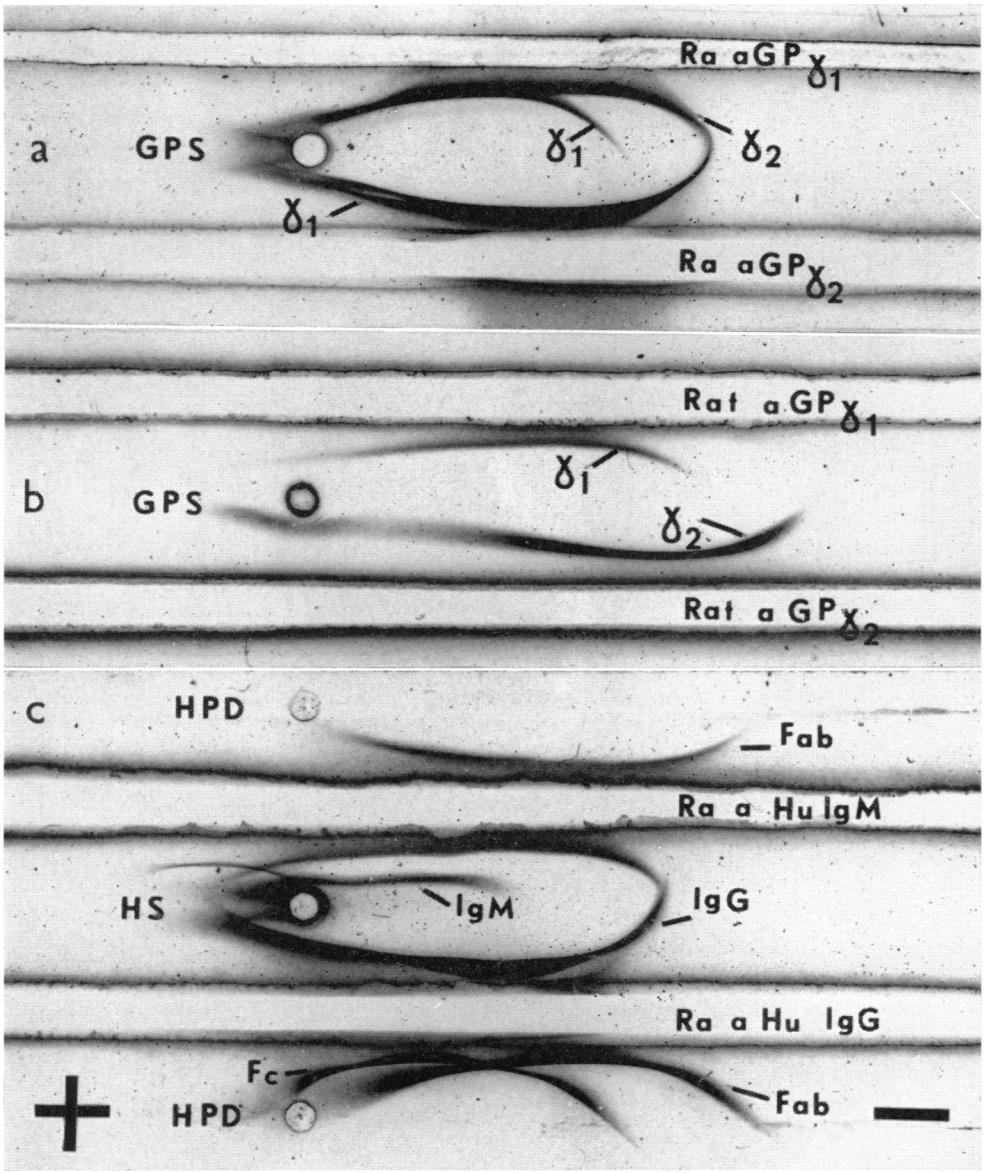

FIG. 2. Specificity of rabbit (Ra) and rat antisera against guinea-pig (GP) γ_1- and γ_2-globulins, and of rabbit antisera against human (Hu) IgM and IgG. GPS, guinea-pig serum; HS, human serum; HPD, papain digest of human IgG.

and (b) present a comparison between rat and rabbit antisera which clearly shows the difference of the antibodies made by these two species.

In Fig. 2(b) the specificity of the antisera obtained in rats by immunization with guinea-pig γ_1- or γ_2-globulins is also shown. Owing to the absence of anti-Fab antibodies

both antisera give only one arc of precipitation with the corresponding immunoglobulin, (see Fig. 2a). Immunization of rats with $(Fab)_2$ fragment of guinea-pig γ_2-globulin failed to produce anti-Fab antibodies, while some anti-Fc antibodies were produced against traces of undigested γ-globulin present in the $(Fab)_2$ preparation.

Guinea-pigs immunized with normal human IgG or with rat 7S immunoglobulins produced a substantial amount of anti-Fc antibody, while no antibody was found against the Fab fragment. Fig. 1(c) and (d) show a comparison of guinea-pig and rabbit antisera. It must be mentioned that guinea-pigs immunized with normal rat 7S immunoglobulins produced, in some instances, anti-Fab antibodies. However, the quantity was very small, and slight dilution of the antisera reduced their concentration below the sensitivity of the immunoelectrophoretic technique. On the other hand, the various rat immunoglobulins were clearly defined by guinea-pig antisera, owing to the antigenicity of their Fc fragment and because of the absence of anti-Fab antibodies. In this respect, guinea-pig antisera proved to be very useful for the study of the heterogeneity of rat immunoglobulins (Binaghi and Merlo, 1966).

Immunization of guinea-pigs with human IgM and of rats with guinea-pig IgM also failed to produce any anti-Fab antibodies.

DISCUSSION

It is apparent from these results that the specificity of the antibodies produced upon inoculation with heterologous immunoglobulins in rabbits or guinea-pigs and rats, is quite different. While rabbits, without exception, produced antibodies directed to both Fc and Fab fragments, guinea-pigs and rats only responded with antibodies directed against the Fc fragment.

It must be emphasized that this difference may be limited to the method of immunization employed and to the technique used for the detection of the antibodies. Our results show that, under the conditions described, anti-γ-globulin sera produced in rats and guinea-pigs were essentially free of antibodies directed to the determinants present in the Fab fragment, which is shared by all the immunoglobulins of one animal species. Consequently these antisera were specific for the particular immunoglobulins employed, without further processing.

The use of guinea-pigs and rats to prepare antisera has so far been very limited, partly because these animals are reputed to be poor precipitin producers. In fact they are excellent precipitating antibody producers, not only from the standpoint of the quantity of antibody they produce when adequate schedules of immunization are employed, but also because of the rapidity of the immunological response.

Through some experience in the immunization of rats and guinea-pigs, we have come to the conclusion that these animals are rather selective in the way they respond to immunization with mixtures of antigens. We have frequently observed that immunization with foreign serum, for instance, results in the production of antibodies against only a few of the serum components. This limited response is sometimes very striking and eventually can be very useful for practical purposes. In the experiments described in this paper, this selective response is manifested at the molecular level, providing a simple method to prepare specific immunological reagents.

Various hypotheses can be considered to account for the lack of antibody production in rats and guinea-pigs, against Fab fragments of heterologous immunoglobulins. It may be

that some antibody is actually formed, but in such a small quantity that it cannot be detected by agar diffusion techniques. This is probably what happens when guinea-pigs are immunized with normal rat immunoglobulins: in this case a weak response is observed in some animals and it could be expected that prolonged immunization and repeated boosters would enhance anti-Fab antibody synthesis.

Another possibility is that competition between the antigenic determinants within the molecule is playing a role. Competition of this kind is regularly observed between haptenic groups and protein determinants during immunization with hapten-conjugated proteins, and its quantitative aspects have been evaluated in some systems (Amkraut, Garvey and Campbell, 1966). To test this possibility, rats were immunized with guinea-pig $(Fab)_2$ fragment. Analysis of the antisera obtained indicated that the animals did not produce detectable amounts of anti-Fab antibodies. Moreover, in a series of preliminary experiments, rats immunized in this way were inoculated intravenously with 40 μg/100 g weight of ^{125}I-labelled $(Fab)_2$ fragment and its clearance studied. No differences were observed between the rate of clearance of the labelled antigen in immunized and normal rats. On the other hand, the rate of clearance of ^{125}I-labelled guinea-pig γ_2-globulin was clearly accelerated in rats immunized with guinea-pig γ_2, as compared with normal rats. These experiments indicate that, at least in this case, competition between antigenic determinants within the molecule was not the reason for the lack of production of precipitating anti-Fab antibodies.

It is also possible that the low immunogenicity of some portions of the immunoglobulin molecule is determined by structural similarities between the immunoglobulins of different animal species.

The present observations show that species differences exist in response to heterologous immunoglobulin antigens. Rabbits are able to produce antibodies against immunoglobulin determinants on both Fab and Fc fragments, while the response of rats and guinea-pigs is limited to determinants of the Fc fragment. Analyses of antisera made in horses, sheep and monkeys seem also to indicate some degree of specificity in the response of these species (Fahey and McLaughlin, 1963). It is important to take into account these differences when selecting an animal species for the production of antisera of a particular specificity, and it is very interesting that specific antisera for each class of immunoglobulin can easily be obtained in convenient laboratory animals such as guinea-pigs and rats.

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