Studies on guinea-pig subclass IgG 1 and IgG 2 antibodies

The quantitation of the subclass antibodies in antisera obtained in an early stage of immunization with bovine serum albumin .

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Introduction

It is well recognized that there are two subclasses in guinea-pig immunoglobulin G, one of them is an electrophoretically slow type designated as IgG2, and the other is the faster type designated as IgG1, and the former is a major component in the normal serum and the latter is found only in a low level.

From the data of the amino acid sequence analyses, it is known that these subclass immunoglobulin molecules are quite closer to each other than any other, but are contrasted with each other in the biological or functional properties. For instance, the subclass IgG1 antibodies are required for the homologous anaphylactic reactions, incapable of sensitizing heterologous tissues and fixing complement components in the classical pathway, but the subclass IgG2 antibodies are required for sensitizing heterologous tissues.

It has been also known that when guinea-pigs were immunized with certain antigens emulsified in Freund's complete adjuvant (FCA), the responses are found in both subclass antibodies, but are major in IgG2 and minor in IgG1 at an early course of the immunization, whereas with the antigen in Freund's incomplete adjuvant (FIA), the products are major in IgG1 antibodies. From these facts it has been suggested that the responses of these subclass antibodies are susceptively influenced by the immunization

procedures, particularly by the kinds and/or doses of adjuvant and antigen, and FCA is required for the intensive response of IgG2 subclass antibody.

Furthermore, in the case of anti-hapten antibody, there are some evidences that when guinea-pigs were immunized with DNP-E.coli, regardless with or without of FIA the response of the anti-DNP antibodies are selectively dominant in IgG2 subclass.

Therefore, it has been speculated that a certain sequential or preferential correlation exists between the responses of guinea-pig subclass antibodies.

In our previous experiments on the immunization of tetanus toxoid, it was found that the symptoms of cutaneous anaphylaxis are observed variously in the guinea-pigs in an early course of the immunization under a specified condition. The observation seemed to show that homocytotropic antibodies had been produced in the immunized animals at so early stage. However, the biological roles of the antibodies in defence mechanisms in vivo and of the correlation between the responses of each subclass antibody have not yet understood.

In this paper, in order to investigate further the distinctive biological properties and the distribution in antisera, the separation and quantitation of the subclass antibodies from the individual antisera obtained at various days

in the early course of the immunization with the minimum dose of BSA required for the anaphylactic sensitization were carried out by a modified indirect method of radio-immuno assay using individually specific goat antibodies against the subclass IgG1 and IgG2.

Materials and Methods

Albumins : Bovine serum albumin (BSA, 3 times recrystallized) was obtained from Nutritional Biochemicals Co.(U.S.A.). Guinea-pig serum albumin (GSA) was prepared by saturated ammonium sulfate precipitation and DEAE-Sephadex A-50 column chromatography from the pooled normal sera.

Guinea-pig subclass IgG's and goat antibodies : The isolation and purification of these immunoglobulins and antibodies were performed according to the procedures described in our previous reports. In brief, the sera obtaind from guinea-pigs immunized with BSA in FCA were submitted to the isolation of the subclass IgG's. The procedure consisted of the ion-exchange chromatography on a column of DEAE-Cellulose and the gel filtration on Sephadex G-150 column. Antiserums to these subclass immunoglobulins were obtained by immunizing goats with the isolated IgG1 and IgG2, respectively. The IgG fractions were isolated from these goat antisera according to the same procedures as used in the case of guinea-pig immunoglobulins, except for a little differences in the condition of column chromatography. The isolated antibody preparations were strongly reacted with each corresponding antigen, but still reacted weakly with each other. So, in order to obtained the subclass specific antibody, the antibody preparations were cross-absorbed with each other subclass IgG coupled to CNBr-activated Sepharose 4B. After the repeated absorption,

these preparations did not show any precipitation reaction with the heterologous antigens and the other serum proteins. The specificity of these antibodies will be cofirmed in the radio-immuno assay under Experiments and Results. Utilizing the specific antibodies, the guinea-pig subclass IgG's were further purified by cross absorptions with these antibody immunoadsorbents. These purified antigens and antibodies were used in the following experiments.

Immunoelectrophoresis and double diffusion tests : Immuno-electrophoresis in 1.0% agar gel was done using a veronal buffer pH 8.6, μ;0.05. An electrophoretic rum of 1.5 hours was obtained using 160 V. Double diffusion precipitin tests were done using 1.2% agar dissolved in borate buffer pH 8.0.

Determination of protein : The absorbancy at 280 nm was used for calculating protein concentration. The values of $E^{1.0\%}_{1.0\,cm}$ were taken as 14.1, 14.0 and 7.0 for guinea-pig IgG, goat IgG and BSA, respectively.

Immunization of guinea-pig : Randomly bred Hartley strain guinea-pigs of female, weighing 300 ~ 350 g were used. Aminals were received single intraperitoneal injection of various amounts of BSA mixed with aluminum adjuvant.

Anaphylactic reactions :

Passive cutaneous anaphylaxis (PCA) : Several dilutions of purified IgG1 antibody or test samples (immunized serum from individual bleedings) were injected intradermally in

0.1 ml quantities into shaved dosal of normal guinea-pigs in duplicate. After a short latent period (3 hours) or a long latent period (24 hours) the animals were injected intravenously (i.v.) with 1.0 ml of 0.5 per cent solution of evans blue in saline (0.15 M NaCl) containing 1.0 mg of BSA. Thirty minutes after the antigen injection, the animals were killed and the skin was inverted and the lesion diameter was measured on the outer (or inner) surface of the skin with a slide calipers. The reactions were graded according to the diameter of the colored spots ; 0 = no reaction, 1/2 = less than 5 mm, 1 = from 5 to 10 mm, 2 = from 10 to 15 mm, 3 = from 15 to 20 mm, 4 = more than 20 mm.

Active cutaneous anaphylaxis (ACA) : At first, guinea-pigs were intraperitoneally injected with various amounts of BSA in aluminum precipitates, and at various days after the injection, various amounts of BSA (1.0, 0.1, 0.01 mg) in 0.1 ml volume were intradermally injected to the shaved dorsal region of the actively sensitized guinea-pigs, and at the almost the same time, 0.5 ml of 1.0% evens blue solution was intravenously injected the animal. The skin reactions with blue spots were appeared with in a few minutes and completed with in 30 minutes after the antigen injections. Animals were killed, and the sizes of spots were measured and graded according to the similar way used in the PCA reaction.

Passive systemic anaphylaxis (PSA) : Various amounts of

the purified IgG1 antibody were injected intravenously into normal guinea-pig weighing 300 ~ 350 g. Forty eight hours later, the animals were intravenously injected with 2.0 mg of BSA in 1.0 ml of saline. The reactions were graded by the observed symptoms of anaphylactic shock as follows ; 0 = no reaction, 1 = slight reaction, 2 = moderate reaction, 3 = severe reaction, 4 = death.

Active systemic anaphylaxis (ASA) : The sensitized guinea-pigs as well as the ACA, were intravenously injected with 2.0 mg of BSA in saline, the observed symptoms of the systemic anaphylaxis were graded as in the case of PSA.

Radioactive iodination of proteins : Purified immunoglobulins (guinea-pig IgG1, IgG2 and goat anti-IgG1, -IgG2) and BSA were iodinated by the chloramine T method described by Greenwood et al. (3) by using carrier-free $Na^{125}I$ (New England Nuclear U.S.A.). The solution containing 0.1 ~ 1.0 mg of the protein preparation was mixed with about 2 ~ 20 μCi of ^{125}I, and a small portion of chloramine T freshly dissolved in 0.005 M phosphate buffered saline pH 7.2 (PBS) were added to the reaction mixture, to be a ratio of chloramine T to protein 1 : 20 (w/w). The reaction mixture was allowed to proceed in an ice cold for 10 minutes and then terminated by addition of sodium hydrosulfite (50 μg/ mg of protein). Unbound ^{125}I was removed from the sample by chromatography on a 1.2 x 30 cm column of Bio-gel P-10 (Bio-

Rad Laboratories, U.S.A.) equilibrated in PBS. The labeled samples were throughly dialysed against a lot of PBS. The specific radioactivity bound to protein was determined by 10% trichloroacetic acid (TCA) precipitation of a small portion of the sample and more than 98% of the total counts of labeled material was found in the precipitate.

Experiments and Results

Characterization of goat antibody immunoglobulins : In order to comfirm, the mono-specificity of the purified goat antibodies to the guinea-pig subclass antibodies were tested against these antigens by immunoelectrophoresis and double diffusion in agar gel. The results of the immuno-electrophoretic analyses showed that the each antibody reacted to the corresponding subclass immunoglobulin alone with a single precipitation arc, and never reacted with the other guinea-pig serum proteins including IgG2, respectively. They were further confirmed even by the more sensitive radio-immuno assay, that is, the radioactively labeled antibodies quantitatively bound to the solid phase immunoadsorbent made of the homologous antigen, the percentages of the bound cpm to the total one were 9.0% for the anti-IgG1, and 6.5% for the anti-IgG2, but only less than 0.15% was cross-reactable to the heterologous antigen or guinea-pig serum albumin for the respective antibodies. These percentage values were also used to estimate the specific antibody contents of the anti-subclass antibody IgG.

Characterization of guinea-pig IgG1 and IgG2 immunoglobulins : Because the purified guinea-pig subclass antibodies were prepared from pool of the hyper immunized antisera with bovine serum albumin, these preparations contained an appreciable amounts of anti-BSA antibodies belonging each of

the subclass immunoglobulins. The antibody contents of each preparation could be determined by the direct radio-immuno assay using polystyrene tubes coated with BSA. The radio-activily labeled IgG1 and IgG2 preparations quantitatively bound to the solid phase immunoadsorbent of BSA, and the percentage values of the bound cpm to the total one were taken as the specific anti-BSA activities involved in the subclass immunoglobulins, that is, 41% for IgG1 and 16% for IgG2. The antibody concentrations of the subclass preparations were caluculated from these percentage values.

PCA activity of the purified IgG1 : The IgG1 preparation used for the experiments was containd a known amount of anti-BSA antibody as described just above. Therefore, PCA reactions could be sensitized with this IgG1 and be induced by the challenge of BSA antigen. To determine the minimum dose for passive sensitization and the latent period, normal guinea-pigs were intradermally injected with various amounts of the IgG1, and various hours later the animals were challenged by the procedure described in Methods.
The positive PCA reactions were observed at 3 hours after the injection with more than 0.1 μg of the IgG1 antibody (in 0.1 ml volume). However, at the latent period prolonged to 24 hours, the minimum dose decreased as about one tenth of the above value. The PCA reaction in the following experiments were performed under the condition.

Recently, it is established that guinea-pigs produced two distinct types of homocytotropic antibody, one belong to the IgG1 subclass and the other to IgE class of immunoglobulin and they are functionally distinguishable by their biological properties. In this paper the criteria were applied for the distinction between IgG1 and IgE, that is, IgE antibody loses its ability to induce PCA reaction by heating at 56°C for 2 hours, and persists in the passively sensitized skin for over 10 days, finally IgG1 bound to Protein A from staphylococcus aureus loses its anaphylactic reactivity. The results were obtained that no interference of the anaphylactic reactions elicited the IgG1 preparation by heating was observed and the skin persistence was completely extinguished with in 3 days and the inhibition of the PCA reaction by addition of Protein A was observed.

Passive systemic anaphylaxis (PSA) of IgG1 : To determine the minimum dose of passive sensitization, normal guinea-pigs were injected intravenously with the purified IgG1 in various amounts, and 48 hours later, the anaphylaxis both of fatal systemic and of local reaction to the guinea-pigs were found with the administration of the IgG1 containing 50 μg of the anti-BSA antibody protein, but less than this quantity, only the local reaction was positive and with less than 10 μg of the antibody, any anaphylactic reaction could not be found.

The effect on the anaphylaxis of the IgG1 antibody by

coexisting IgG2 antibody were tested and no effect was found by the addition of over twenty times higher quantities of the IgG2 antibody than that of the IgG1 antibody.

Anaphylactic reactions in the animals sensitized with various dose of BSA : Guinea-pigs were intraperitoneally sensitized in aluminum precipitate with various amounts of BSA and both anaphylactic reactions were investigated in these groups of 4 animals. The weak ACA reactions were observed in the groups immunized with 1.0 μg of BSA at 10 days after the injection, but systemic one did not. The apparent anaphylactic reactions were found in these both groups with 10 and 100 μg of the antigen at least in 15 days after the injection, but the fatal systemic one did not, and at 20 days later, the fatal or severe systemic reaction were observed in these two groups.

Determination of the subclass antibodies in the sensitized animal sera : It was considerably hard to quantify a very minute amount of antibody by the conventional techniques, furthermore the procedure had to be able to determine each quantity of the specific subclass antibodies using a small volume of the antisera. From these reasons, the radio-immuno assay with some modifications has been successfully applied to this purpose. The procedures of the indirect radio-immuno assay consist of three reaction steps as follows, 1) coating polystyrene tubes with BSA, 2) binding anti-BSA

antibodies in guinea-pig antiserum, finally, 3) binding radio actively labeled anti-guinea-pig subclass antibodies specific for each of the subclass IgG's. The practical procedures would be described in more detail. One ml portions of 1.0% BSA solution were pipeted into polystyrene tubes and stood for 1 hour at room temperature. The solutions were aspirated and then washed 3 times with PBS. To block the remaining vacant site, the tubes were filled with 1.0% solution of GSA in PBS, and allowed to stand for 1 hour at room temperature, then the GSA solution was aspirated and washed 3 times with PBS.

The second, various amounts of the primary antibodies (guinea-pig anti-BSA IgG1 and IgG2) and 0.1 ml portion of GSA solution were added to the tubes, the tubes were incubated for 24 hours at room temperature with rotatory stirrer, the solution was removed and washed 3 times with PBS. Finally, ^{125}I-labeled secondary antibody (goat anti-guinea-pig IgG1 and IgG2) and 0.1 ml portions of 1.0% GSA solution were added to the primary antibody bound tubes, respectively. The tubes were incubated for 24 hours at room temperature by rotation, washed 3 times with PBS and then the remaining radio activities in tubes was counted with auto-gamma scintillation spectrometer (Model 5320, Packard U.S.A.). The correlations between the quantities of the subclass antibodies and the radioactivity of the specific antibodies against these subclass IgG's were obtained.
The quantitating reference curves for each subclass antibody

were used for the actual determinations of the antibodies in the antiserum obtained in an early stage of the immunizations.

In order to investigate the sensitivity and accuracy of this method the amounts of the primary antibodies bound to the antigen coated tubes were directly determined using each of the ^{125}I-labeled subclass IgG's in various concentrations. In separate experiments up to 1.5 μg of the primary antibodies were quantitatively bound to the tubes in both cases, but with more than this quantity, the bindings were gradually saturated and the quantitativeness was unestablished in the both cases. The binding rates of the antibodies were also determined at various incubation hours in these measurments, and it was found that more than 90% of the ^{125}I-labeled antibodies were able to bind on the coated tubes within a few hours.

To determine the optimal concentration of the ^{125}I-labeled secondary antibodies, these antibodies were added to the tubes combined with given amounts of the radioactively non labeled IgG1 and IgG2. At two different concentrations (200 and 400 ng) of the antibodies, the amounts of the bound primary antibodies in both cases, and the reference curves for the determinations of each subclass antibody were obtained, and the measurable ranges of the assay were found to be 1 to 20 ng of the antibodies.

Under the coditions determined in the above reactions, the guinea-pigs immunized with 100 μg of BSA in aluminum precipitates

were bled from the ear vein in various days after the immunization. The result indicated that both IgG1 and IgG2 antibodies were concomitantly detectable in the serum obtained on the 10th day after the immunization. In 15th day, it was found that the most of the increase of antibody contents was due to the production of IgG1 antibody, and the concentration of IgG2 antibody was gradually increased.

PCA activity of antiserums of individual bleedings at early stage of immunization : To examine PCA activity of the antiserum obtained in the early stage of immunization, normal guinea-pigs were injected intradermally with the test antiserum in a various dilutions, and 24 hours later the animals were challenged by intravenous injection of 1.0 mg BSA in 0.5% evans blue solution. The results shown that the degree of PCA reactions were in proportional to their antibody concentration determined above.

Discussion

It is well known that antibody responses are susceptively influenced by immunization procedures. In the case of guinea-pig immunization, the responses of subclass IgG1 and IgG2 antibodies to certain antigens are affected with the kinds of antigens and adjuvants used for the immunization. (1, 4 ~ 7)

Recently, it has been shown by Furuichi et al. (2) that when guinea-pigs were immunized by DNP coupled E.coli, regardless with or without of Freund's incomplete adjuvant, the production of anti-DNP antibody is selectively dominant in IgG2 subclass.

From these results, it has been speculated by them that a certain sequential or preferential correlations would be involved in the responses of these subclass antibodies, as well as in the case of the responses of subclass antibodies in human and murine immunoglobulins.

However, the results of the determination of each quantity of the subclass antibodies in individual antiserum indicate that appreciable amounts of both IgG1 and IgG2 antibodies are concomitantly produced in an early stage of the immunization. And also, actually, it has confirmed by the antisera obtained in a exceedingly early stage of the immunization and any appreciable amount of the other reaginic antibody IgE could not detected functionally in these antisera

by the distinguishable properties of these homocytotropic antibodies, such as the heat stability and skin persistency.

The possible conclusion to be drawn from the present study is that each of the subclass antibodies are independently synthesized from separate cells committed to the given antigen and no sequential response are found in these subclass antibodies, as well as in the case of human IgG subclass antibodies.

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