ELECTROPHORETIC SEPARATION AND PROPERTIES OF MOUSE ANTIHAPTEN ANTIBODIES INVOLVED IN PASSIVE CUTANEOUS ANAPHYLAXIS AND PASSIVE HEMOLYSIS*

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Purified mouse antibodies isolated from peritoneal exudates of hyperimmune animals and directed against haptens or foreign proteins were found on immunoelectrophoresis to consist, regardless of specificity, of two families of molecules which migrate in the γ-region with small differences in mobility, and which possess similar as well as distinct antigenic determinants (1). The relationship of these two antibody types with the mouse β_{2A}-globulins was not investigated, but their electrophoretic mobility indicated that they should both be considered as γ–antibody globulins.

Guinea pig 7S antibodies against a single specificity have also been found to consist of two families of antibodies, γ_1 and γ_2, with different electrophoretic mobilities (2). These two antibody types differed antigenically only in their F fragment (piece III of Porter) (3). They have been shown to mediate different biological activities in the guinea pig, the faster migrating γ_1-antibodies being responsible for anaphylactic sensitivity (4), and the γ_2-antibodies for such phenomena which depend upon the fixation of hemolytic complement such as cell lysis and the Arthus reaction (5). These findings suggested that in the mouse different antibody types may similarly mediate different biological activities.

In the present study, several biological properties of mouse-purified antihapten antibodies have been investigated: the capacity to transfer passive cutaneous anaphylaxis (PCA) in the mouse (6), the capacity to transfer PCA to the guinea pig (7), and the ability to provoke passive hemolysis of antigen-coated tanned sheep red blood cells in the presence of complement. The data indicate that, as in the case of the guinea pig, mouse γ_1-antibodies transfer anaphylactic sensitivity in the species, while mouse γ_2-antibodies appear to be responsible for passive hemolysis in the presence of complement. The capacity to transfer

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PCA to guinea pig is a property of the slowest migrating fraction of the γ_2-antibodies. The availability of specific antisera against mouse β_{2A}-myeloma proteins (8) allowed the mouse γ_1-antibodies to be distinguished antigenically from β_{2A}-globulins. The sensitivity of the various biological properties of mouse antibodies to reduction and alkylation was also investigated.

Materials and Methods

Animals.—Random-bred adult Swiss Webster mice were used for immunization and for PCA. Hartley strain guinea pigs were used for PCA.

Antigens.—Two protein conjugates, 2:4-dinitrophenyl bovine gamma globulin (DNP-BGG) and *para*-iodobenzenesulfonyl bovine gamma globulin (pipsyl-BGG) were used for immunization. 2:4-Dinitrophenyl bovine serum albumin (DNP-BSA) was used to coat sheep red blood cells (SRBC) in order to detect agglutinating and lytic anti-DNP antibodies. These antigens were all highly conjugated and prepared according to previously described techniques (9).

Immunization.—The mice were hyperimmunized to yield high titer antibody in their peritoneal fluid according to the technique of Munoz (10). The animals were injected intraperitoneally with 1 mg of the antigen, emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories Inc., Detroit) (total volume injected 1 ml). Booster injections of smaller amounts of antigen were given at weekly intervals for 2 or 3 months.

Preparation of Purified Antibodies.—Antibodies were isolated from the ascitic fluids either from individual mice after successive tappings, or from pools obtained from several animals of a same group. The isolation procedures after decomplementation have been described previously (1).

Immunoelectrophoresis.—A modification of Scheidegger's technique (11) for agar gel immunoelectrophoresis of the purified mouse antibody preparations was used. The slides were developed with a rabbit serum against mouse serum, R1. Two rabbit antisera against mouse β_{2A}-myeloma globulin were also used: serum RPC6 prepared by Dr. E. Osserman of Columbia University and serum R52A prepared by Dr. John Fahey of the National Institutes of Health. We are grateful to Dr. Osserman and Dr. Fahey for the use of these antisera.

Electrophoretic Separation of Different Antibody Fractions.—Electrophoretic separation in agar gel of two mouse anti-DNP antibody preparations, pool I and pool II, and the elution of the fractions were performed as described (12). The electrophoresis was carried out for 3½ to 4 hours at 30 ma and 120 volts.

0.5 cm agar strips were then cut, starting at the anode, 4 cm from the origin. The strips were frozen for 30 minutes in an alcohol-solid CO_2 bath, rapidly thawed at 37°C, and broken with a glass rod. After addition of 1 ml saline to each fraction, the protein was allowed to diffuse for 18 hours at room temperature. The supernatants obtained after high-speed centrifugation were tested for the different antibody activities. A preparation of mouse anti-pipsyl antibodies isolated from a pool of peritoneal fluids obtained from mice immunized with pipsyl-BGG was separated by zone electrophoresis on a starch block (13) using 48 ma and 800 v for 25 hours. Half-inch cuts were extracted with saline. The fractions were dialyzed against dilute phosphate buffer, pH 7.6, and tested for their capacity to transfer anaphylactic sensitivity to mice or guinea pigs.

FIG. 1. Immunoelectrophoretic patterns of purified mouse antibody preparations developed with a rabbit serum anti-mouse serum R1. *A*, anti-DNP antibodies isolated from individual mice. *B*, anti-DNP and anti-pipsyl antibody preparations isolated from pools of mouse ascites fluid.

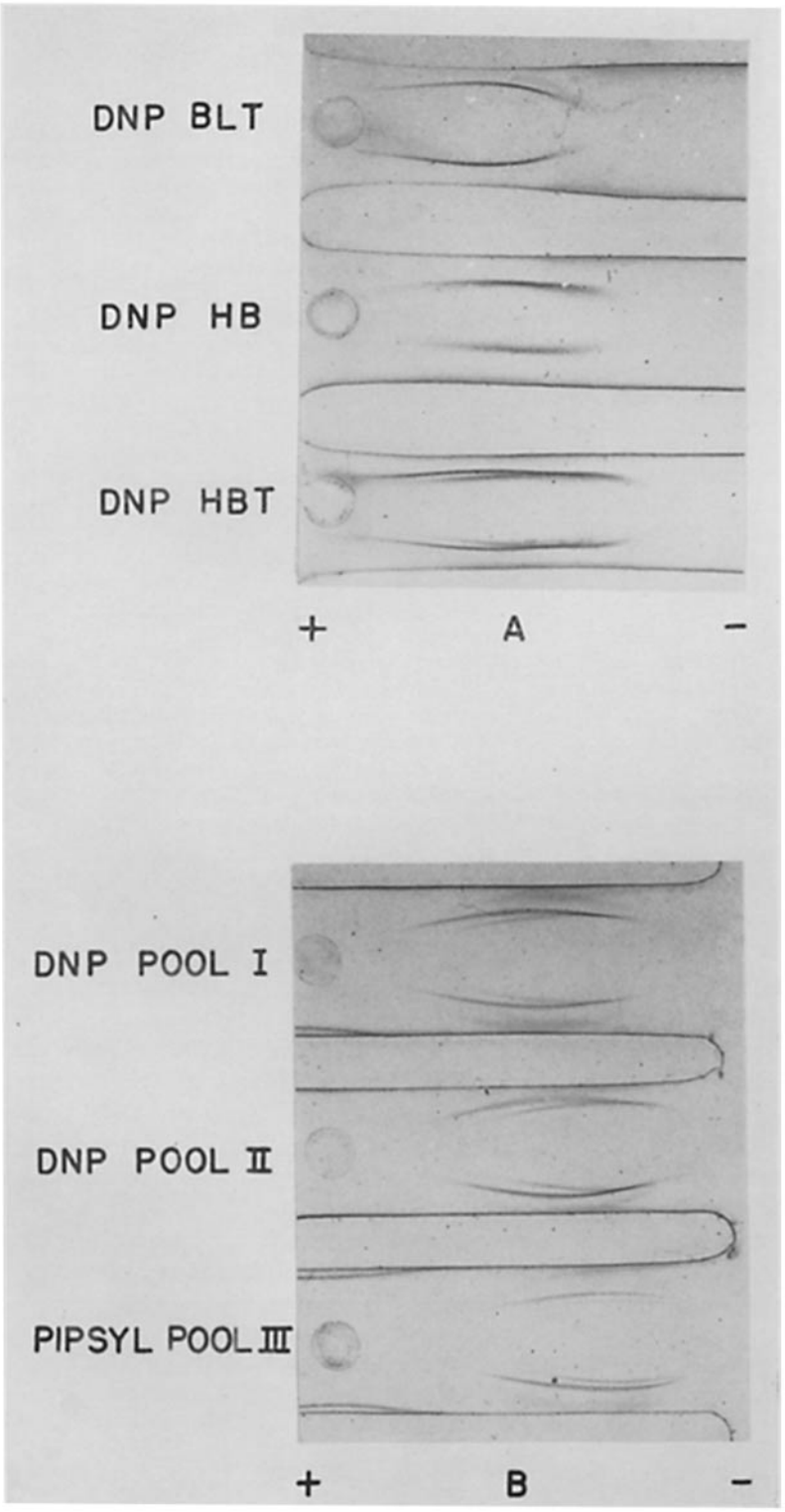

FIG. 1

Passive Cutaneous Anaphylaxis in Mice.—PCA was performed as described by Ovary (6). 0.05 ml of the various dilutions were injected intradermally. Two hours after the sensitizing injections, the mice received intravenously 0.5 ml of a mixture of equal parts of antigen solution, DNP-BSA or pipsyl-BSA (1 mg/ml), and 1 per cent Evans blue dye. The reactions were examined on the inner side of the skin, 10 to 15 minutes after the intravenous injection. Three or four mice received each sample dilution.

Passive Cutaneous Anaphylaxis in the Guinea Pig.—PCA was performed as described (7) using 500 μg of DNP-BSA or pipsyl-BSA as antigens. Each sample dilution was tested in 3 guinea pigs.

Agglutination Techniques.—Tannic acid-treated sheep erythrocytes were prepared for passive hemagglutination according to the technique described by Stavitsky (14). These cells were coated with DNP-BSA by incubating them for 30 minutes at room temperature with a 0.4 mg/ml solution of antigen. The agglutination test was made in a total volume of 0.6 ml using 0.1 ml of 0.5 per cent coated cells and 1 per cent of normal absorbed rabbit serum. Serial dilutions in twofold steps were made of all the antibodies or fractions tested. Controls of the antibody dilutions with tanned uncoated cells were used. The tubes were incubated for 18 hours at 4°C.

Hemolysis.—The technique was similar to the passive agglutination, and frequently was done on the same tubes after reading the agglutination titers. In this case, veronal buffer was used as diluent, and 0.1 ml of a 1:3 dilution of normal mouse serum was added as source of complement. The cells were then incubated for 30 minutes at 37°C.

The highest antibody dilution showing 100 per cent lysis was considered the hemolytic titer. Passive agglutination and lysis when repeated gave very reproducible results.

Reduction and Alkylation.—Mercaptoethanol was added to the purified antibody solutions to make a final concentration of 0.1 M. The solutions were incubated at room temperature for 2 hours and dialyzed against either iodoacetamide (0.02 M) in buffered saline, or buffered saline (pH 7.6). The excess of iodoacetamide was eliminated by further dialysis against saline.

Controls were similarly treated, without adding mercaptoethanol.

RESULTS

Immunoelectrophoresis of Purified Mouse Antihapten Antibodies.—Purified anti-DNP or anti-pipsyl antibodies isolated from the ascites fluids of individual mice or from pooled ascites fluids were analyzed by immunoelectrophoresis using a rabbit antiserum against mouse serum. Most of the antibody preparations presented two precipitin lines in the gamma region, indicating the presence of two antibody types, with small differences in their electrophoretic mobilities (Fig. 1). Among the many preparations studied, two anti-DNP antibodies from individual mice showed only one of the two antibody components, BLT containing only the faster migrating antibody type and HB containing nearly exclusively the slower migrating antibody type.

In order to distinguish these two antibody types from the mouse β_{2A}-globulins, immunoelectrophoresis of mouse serum and anti-DNP and anti-pipsyl antibody

FIG. 2. Comparison of the immunoelectrophoretic patterns of mouse serum β_{2A}-globulins and of purified mouse antibodies developed with a rabbit serum anti-mouse serum R1 and with two different rabbit anti-sera against mouse β_{2A}-myeloma globulins before and after absorption with mouse anti-pipsyl antibodies pool III.

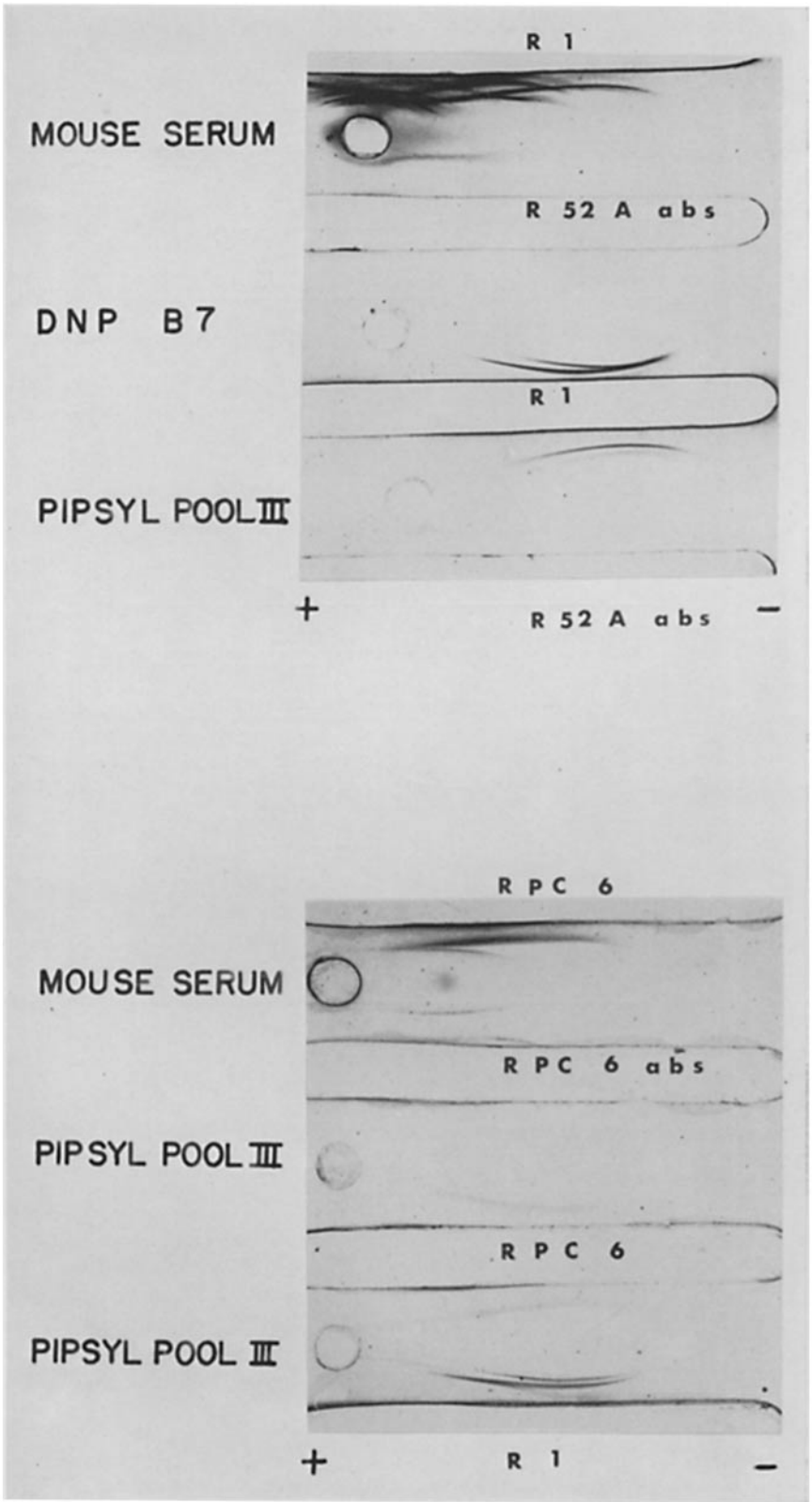
R 1
MOUSE SERUM
R 52 A abs
DNP B7
R 1
PIPSYL POOL III
+
R 52 A abs
−
RPC 6
MOUSE SERUM
RPC 6 abs
PIPSYL POOL III
RPC 6
PIPSYL POOL III
+
R 1
−

FIG. 2

preparations containing both antibody types were developed with two rabbit antisera prepared against mouse β_{2A}–myeloma globulins (Fig. 2). The mouse

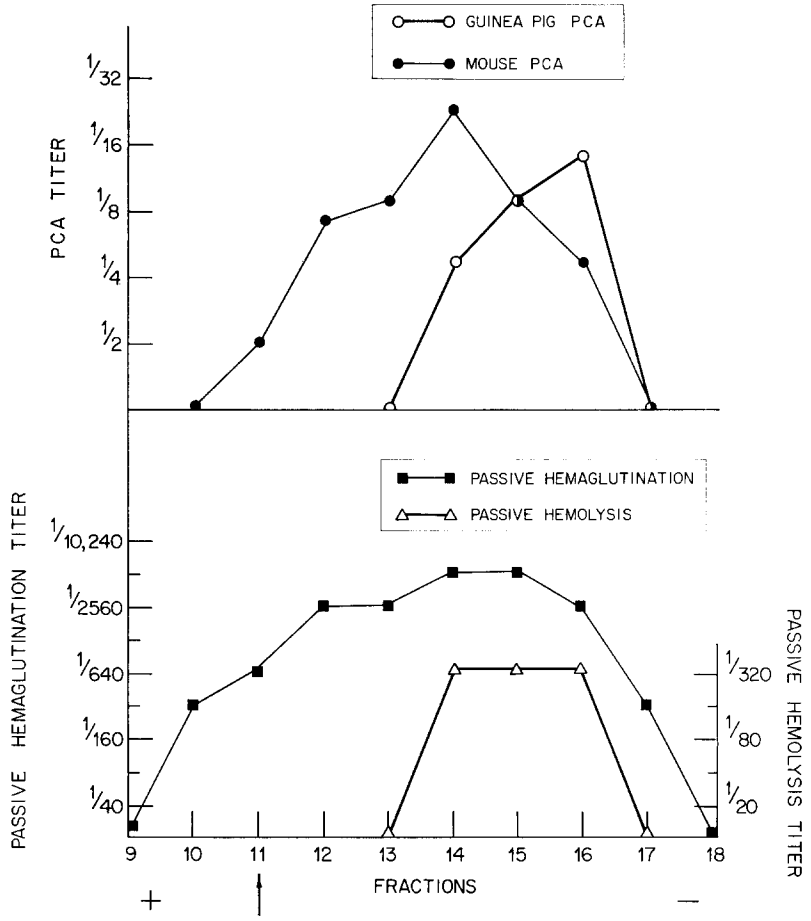

FIG. 3. Zone electrophoretic separation in agar gel of a mouse anti-DNP antibody pool (pool I) showing in the upper part the PCA titers in mice and guinea pigs and in the lower part a comparison of passive hemagglutination and passive hemolysis titers of the individual fractions. (Immunoelectrophoretic pattern of anti-DNP pool I in Fig. 1.)

β_{2A}-line can be clearly identified in immunoelectrophoretic patterns of whole mouse serum developed with either anti-β_{2A}-globulin sera. One of the antisera, RPC6 unabsorbed, reacts well also with mouse γ-globulins. This line disappears completely when the antiserum is absorbed with purified mouse anti-pipsyl antibodies (pool III). The β_{2A}-line remains unchanged after absorption of both antisera with a preparation of purified mouse antibodies (anti-pipsyl pool III). The

β_{2A}-line was never observed in immunoelectrophoresis of purified anti-DNP or anti-pipsyl antibodies which contained the two antibody types described above. These experiments show that the two antibody types present in the prepara-

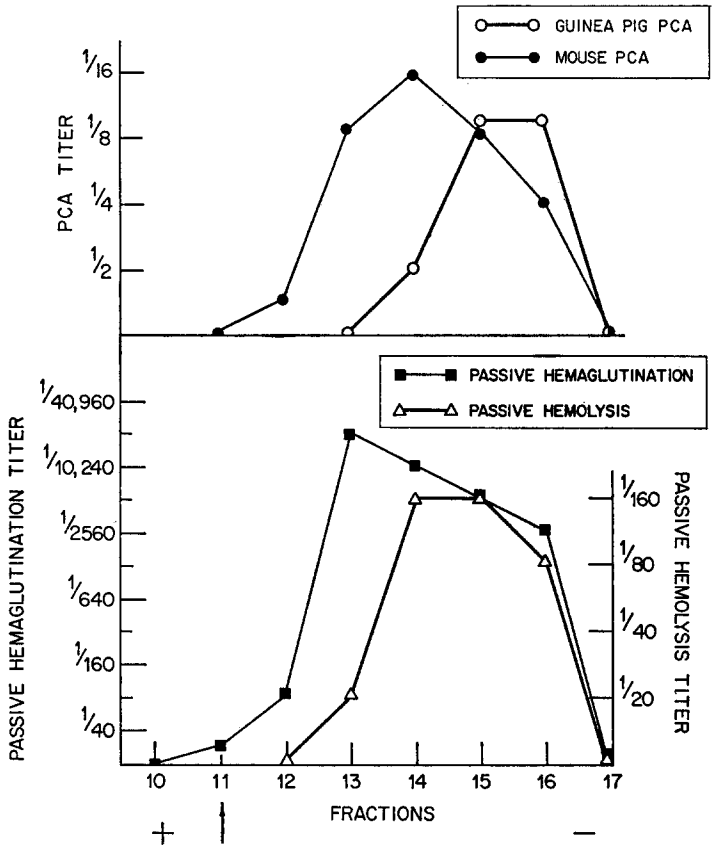

FIG. 4. Zone electrophoretic separation in agar gel of a mouse anti-DNP antibody pool (pool II) showing in the upper part the PCA titers in mice and guinea pigs and in the lower part a comparison of the passive hemagglutination and passive hemolysis titers of the individual fractions. (Immunoelectrophoretic pattern of anti-DNP pool II in Fig. 1.)

tions of purified mouse antibody studied are distinct antigenically and electrophoretically from mouse β_{2A}-globulin. Thus, mice, similarly to guinea pigs, produce two γ-antibody types which we will refer to also as γ_1 and γ_2.

Biological Activities of Purified Mouse Antibodies.—All the anti-DNP antibody preparations studied showed high agglutination titers against DNP-BSA-coated tanned SRBC. Most antibody preparations showed also hemolysis of these cells in the presence of mouse complement. If, instead of mouse serum as a

source of complement, guinea pig serum was used, no hemolysis was observed in this system, even with large excess. However, when in addition to mouse serum, the system was supplemented with guinea pig complement, hemolysis was more rapid and a little more extensive. In every instance the passive hemolysis titers were much lower than the passive agglutination titers. Thirty to 100 times more antibody was required for 100 per cent lysis than for passive hemagglutination.

In an attempt to explore which of the two antibody types, γ_1 or γ_2, is respon-

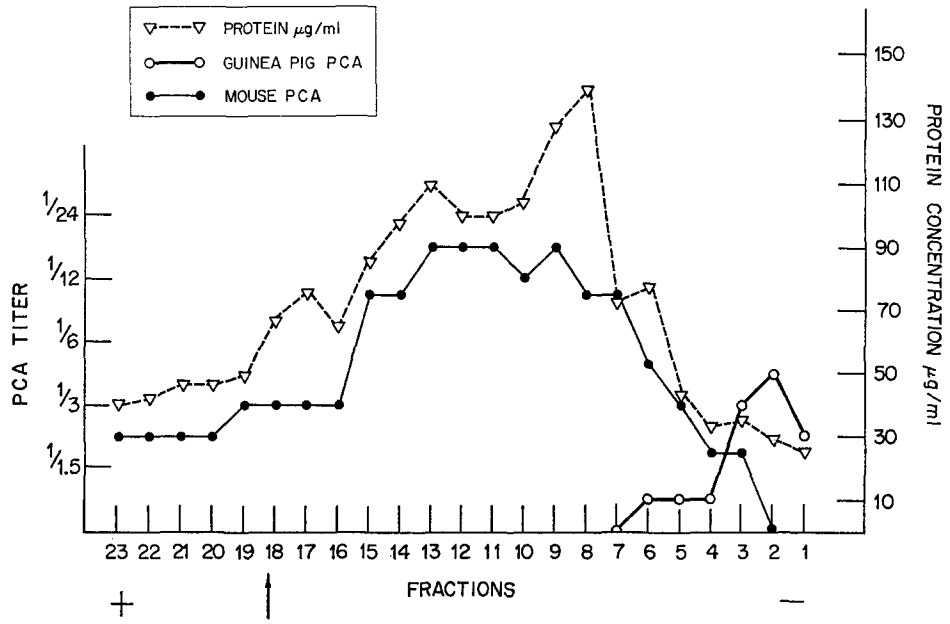

FIG. 5. Starch block electrophoretic separation of a mouse anti-pipsyl antibody pool (Pool III) showing the protein concentration and the respective PCA titers for mice and guinea pigs of the individual fractions.

sible for passive hemolysis and which is capable of transferring anaphylactic sensitivity in the mouse, use was made of the two antibody preparations described above which contained only one of the antibody types. Anti-DNP preparation BLT which contained only γ_1-antibodies did not cause passive hemolysis of DNP-BSA–coated tanned SRBC, but was capable of transferring PCA in the mouse. This same preparation could not sensitize guinea pigs for PCA even when as much as 8 μg per site was used. All anti-DNP preparations showing a strong γ_2-line in immunoelectrophoresis were able to hemolyze passively sensitized SRBC and to transfer PCA to guinea pigs. Antibody preparation HB, which contained only the slow migrating γ_2-component, hemolyzed DNP-BSA–coated SRBC.

From these limited observations it would appear that mouse γ_1-antibodies are responsible for anaphylactic sensitivity in mice while mouse γ_2-antibodies possess lytic activity in the presence of complement and can also transfer PCA to guinea pigs. To confirm these findings an attempt was made to fractionate two anti-DNP mouse antibody preparations, pool I and pool II, isolated from pools of ascites fluid, by zone electrophoresis in agar gel. All fractions were tested for passive hemagglutination and passive hemolysis of DNP-BSA–coated SRBC, for PCA in mice, and for PCA in guinea pigs. The results of these experiments are presented in Figs. 3 and 4. In both experiments the agglutination titers follow closely the protein concentration of the fractions. This is not the case for the other activities investigated, although their complete separation could not be achieved. Passive hemolytic activity was present only in the slower migrating fractions; this was also true for the capacity to passively sensitize guinea pigs for PCA. The capacity to confer anaphylactic sensitivity in the mouse extended clearly to the faster migrating fractions where only γ_1-antibodies are found.

A preparation of anti-pipsyl mouse antibodies isolated from a pool of ascites fluid obtained from mice immunized with pipsyl-BGG was fractionated by zone electrophoresis on starch block. The protein concentration as well as the PCA activity for mice and for guinea pigs of the various fractions are shown in Fig. 5. Passive hemagglutination and passive lysis were not performed because coating of SRBC with pipsyl-BSA was not successful. The mouse anti-pipsyl antibodies capable of sensitizing guinea pigs for PCA are present only in the slowest migrating fractions, whereas the antibodies capable of transferring anaphylactic sensitivity to mice, which predominate in this preparation, are absent in the same fractions but extend over the rest of the fractions. Immunoelectrophoretic analysis of the individual fractions revealed that fractions 1 to 4 showed only one slow γ_2-line. The line corresponding to the fast migrating component appeared in fraction 5. Both lines are present in fractions 5 to 18. Fraction 18 and faster migrating fractions showed only the faster migrating γ_1-component.

These experiments indicate that the capacity to transfer anaphylactic sensitivity in mice is a property of mouse γ_1-antibodies. γ_1-Antibodies appear to lack the capacity to cause passive hemolysis in the presence of complement, which is a property of the γ_2-antibody type. The capacity to sensitize guinea pigs for PCA seems to be a property only of the mouse γ_2-antibodies. In the case of the anti-pipsyl antibodies only the slowest migrating fraction of the γ_2-antibodies had this property.

The Effect of Reduction and Alkylation on the Biological Activities of Mouse Antibodies.—Treatment with mercaptoethanol and iodoacetamide did not affect appreciably the capacity of mouse γ_1– and γ_2–anti-DNP antibodies to combine with antigen. There was little change in the passive hemagglutination titer before and after treatment, but the capacity to cause passive hemolysis was completely destroyed by reduction and alkylation. The capacity of mouse anti-

bodies to transfer PCA in the mouse was very much reduced by treatment with mercaptoethanol and iodoacetamide (Tables I and II). This property was not affected by treatment with mercaptoethanol or iodoacetamide alone.

The ability of these antibodies to transfer PCA in the guinea pig was only partially destroyed; a considerable amount of activity remained, showing that this property of mouse antibodies is more resistant to reduction and alkylation

TABLE I

Effect of Reduction and Aklylation on PCA Titers of Mouse Anti-DNP Antibodies

Pool I anti-DNP treated with:	Protein Concentration	Average PCA Titer	
		Mice	Guinea pigs
	μg/ml		
Saline	740	1/32	1/22
Mercaptoethanol	761	1/64	1/22
Iodoacetamide	610	1/32	1/22
Mercaptoethanol and iodoacetamide	720	1/4	1/15

TABLE II

Effect of Reduction and Alkylation on PCA Titers of Mouse Anti-Pipsyl Antibodies

Pool III anti-pipsyl Treated with:	Protein concentration	Average PCA titer	
		Mice	Guinea pigs
	μg/ml		
Saline	250	1/90	1/8
Mercaptoethanol	250	1/90	1/10
Iodoacetamide	360	1/90	1/10
Mercaptoethanol and iodoacetamide	370	1/5	1/5

than either the capacity to provoke passive hemolysis or to transfer anaphylactic sensitivity in the mouse.

DISCUSSION

Antihapten antibody preparations, isolated from the peritoneal fluids of hyperimmunized mice consist of two gamma antibody types, with small differences in electrophoretic mobilities. Immunoelectrophoretic analysis of these antibodies performed with rabbit antisera prepared against mouse β_{2A}-globulins showed that they can be distinguished electrophoretically and antigentically from mouse β_{2A}-globulins. By analogy with the guinea pig where similar antibody types have been described (2), these mouse antibodies have been called γ_1- and γ_2-globulins.

Studies made with antibody preparations containing only γ_1- or γ_2-antibodies and also with antibody fractions separated by zone electrophoresis showed that mouse γ_1- and γ_2-antibodies directed against the same antigenic determinant appear to mediate different biological reactions, as was observed in the case of guinea pig γ_1- and γ_2-antibodies. Mouse γ_1-antibodies are capable of sensitizing mice for PCA and cannot hemolyze antigen-coated sheep erythrocytes in the presence of mouse complement. The ability to cause passive lysis of antigen-coated sheep red blood cells seems to be the property of preparations containing mouse γ_2-antibodies. It can be assumed, therefore, that mouse γ_2-antibodies but not γ_1-antibodies are able to bind complement in the presence of antigen. Passive cutaneous anaphylaxis in the mouse as in the guinea pig requires a latent period for sensitization, which is believed to be the time necessary for the sensitizing antibodies to become bound to tissue receptors (6). The data would indicate that only mouse γ_1-antibodies possess the receptors for fixation to mouse tissues.

Complete electrophoretic separation of mouse γ_1- and γ_2-antibodies was not achieved, as had been done in the case of the corresponding guinea pig antibodies, because the difference in electrophoretic mobility between these two mouse antibody types is very small. Nevertheless an effective separation of the capacities of mouse anti-DNP antibodies to transfer PCA in the mouse and to provoke passive hemolysis was achieved by electrophoresis in agar gel.

The capacity to transfer PCA to guinea pigs with mouse antibodies is a property of γ_2- and not of γ_1-antibodies. The experiments performed with the fractions of mouse anti-pipsyl antibodies separated by starch block electrophoresis indicate, however, that not all mouse γ_2-antibodies, but only the slowest migrating γ_2-fraction can sensitize guinea pig for PCA. The mouse antibody type capable of sensitizing guinea pigs is not the antibody type able to transfer anaphylactic sensitivity in the mouse. Similar observations have been made with antibodies from other species capable of sensitizing guinea pigs for PCA. Rabbit, human, dog, and monkey antibodies which can transfer PCA to the guinea pig are all γ_2, complement-fixing antibodies which are not able to transfer anaphylactic sensitivity in their own species (15). Various animal species,—guinea pig, mouse, rat, human, dog, and monkey,—each produce an antibody type capable of binding to its own tissue receptors to mediate anaphylactic sensitivity in its respective species. But there are apparently different receptor systems involved for each species in their tissues and anaphylactic antibodies. It is of interest that when one crosses species as is done when guinea pigs are sensitized passively with antibodies from other species, the receptors capable of binding to guinea pig tissues to transfer anaphylactic sensitivity to guinea pigs are present only in the γ_2-antibodies which are not sensitizing for the original animal species.

Another point deserving comment concerns the antibody type responsible for anaphylactic sensitivity in each species. γ_1-Antibodies are responsible for anaphylactic reactivity in guinea pigs and mice. Mouse γ_1-antibodies have been

shown to be distinct from mouse β_{2A}-globulins. Guinea pig γ_1-antibodies should not be considered β_{2A}-globulins because human and mice β_{2A}-globulins are characterized by a high carbohydrate content (16), and guinea pig γ_1-antibodies have been found to have the same low hexose content as γ_2-antibodies (17). The anaphylactic antibodies of the rat (18), and of the dog (19) migrate ahead of the main γ_2-components but have not been isolated pure to allow identification either as a β_{2A}- or γ_1-globulin. The human skin-sensitizing reagin although not isolated pure has been reported to be a β_{2A}-globulin. This conclusion has been drawn from studies of inhibition of passive cutaneous sensitization with human β_{2A}-globulins (20) and from reports of absorption of reaginic activity from human sera by anti-human β_{2A}-globulin antisera (21).

The demonstration in the mouse of a γ_1-antibody type which cannot hemolyze passively sensitized sheep erythrocytes raises the question that the presence of such an antibody type in large amounts may interfere with cell lysis by γ_2-antibodies with the same immunological specificity as was shown in the case of guinea pig γ_1- and γ_2-antibodies (22). Since the mouse is the animal species where the immunological enhancement of tumor growth has best been demonstrated, the role of such an antibody capable of blocking cell lysis has to be considered in attempts to explain these puzzling phenomena.

Reduction and alkylation of mouse antibodies nearly completely destroy their capacity to provoke passive hemolysis of sensitized sheep cells and to transfer anaphylactic sensitivity in the mouse but do not affect appreciably their ability to agglutinate these cells. The sites for complement fixation of mouse γ_2-antibodies and for fixation to mouse tissues of mouse γ_1-antibodies are damaged therefore by the irreversible splitting of susceptible SS bonds. Reduction and alkylation of human and rabbit gamma globulin antibodies have been shown to destroy also their ability to fix complement (23).

SUMMARY

Some properties of mouse antibodies produced by hyperimmunization with conjugated haptens, emulsified in complete adjuvant, have been investigated. Under these conditions two antigenically different types of precipitating antibody, with small differences in their electrophoretic mobilities, both migrating in the γ-region, are produced. Both antibody types were shown to be different from the β_{2A}-globulins, as revealed by the absence of reaction with specific rabbit antisera against mouse β_{2A}–myeloma globulins.

γ_2, or the slower migrating antibodies, similarly to what has been described for the guinea pig, were able to provoke lysis of antigen-coated tanned sheep erythrocytes, in the presence of complement. This activity could not be detected in the faster migrating antibody type. The slower migrating fractions of the γ_2-antibodies were able to transfer passive cutaneous anaphylaxis (PCA) in the guinea pig, but not in their own species.

γ_1-Antibodies were found to be capable of sensitizing the mouse for cutaneous anaphylaxis.

The sensitivity of these antibodies to reduction and alkylation was different, the lytic activity being practically abolished, the PCA titers in mice much reduced, and the PCA activity in guinea pigs only partially inactivated.

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