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SERUM-SICKNESS IN RABBITS

VI. INFLUENCE OF REMOVAL OF LIPIDS FROM SERUM ON OCCURENCE OF SERUM SICKNESS[1]

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The studies here reported relate primarily to the rôle that the lipids play in the causation of serum-sickness in rabbits. The word lipid is used as an inclusive term embracing all those substances removed from serum by commonly employed solvents such as alcohol, ether and similar materials. We have previously been led to the conclusion that while the pseudoglobulin of horse-serum is the most important of the 3 major serum proteins in causing serum-sickness in rabbits (1), it seems probable that it is not the pseudoglobulin as such which is the factor responsible for the occurrence of the disease. Our attention was therefore directed towards the possible significance of serum-lipid in causation of serum-sickness. We have therefore exposed whole horse-serum or horse-serum pseudoglobulin to various treatments with several commonly used lipid-solvents and have injected such sera into rabbits in order to determine the occurrence of serum-sickness. It seemed probable that by use of ordinary lipid-solvents it should be possible to remove from serum, active in producing serum-sickness, certain antigenic substances, e.g., the Forssmann heterophilic antigen, and thus to determine the relation of this antigen to serum-sickness in rabbits. Such procedures appeared to be relatively simple since it is possible to treat considerable amounts of serum at room-temperature with alcohol or acetone (2).

We have previously reported that single large injections (5 to

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7 cc. per kilogram) of serum or of the pseudoglobulin (150 mgm. pseudoglobulin-nitrogen per kilogram body-weight) into rabbits, will cause in many instances the appearance of a serum-sickness reaction, evident upon the ears 4 to 7 days after injection (1, 3). The reaction is characterized by the appearance of erythema (morbilliform or scarlatinal) with or without edema, which persists for one, 2 or 3 days and disappears without residual injury.

In addition to the erythema and edema which are particular features of the reaction, we have noted in some of the reacting animals and in some of the animals failing to exhibit the more or less characteristic erythematous reaction, numerous petechial hemorrhages, distributed along the sides and across the tip of the ear. These hemorrhages appear late or almost at the termination of the reaction, are evident for a day or so, become brownish, fade perceptibly and disappear without apparent residual injury to the tissues.

In the studies here reported experiments have been carried out with 4 lots of horse-serum pseudoglobulin and 2 lots of whole horse-serum, portions of each lot being prepared for injection by extraction with lipid-solvents in various manners; in all, 31 preparations having been made from the 6 lots of serum. A certain number of experiments have also been performed in which a certain amount of lipid-free protein was mixed with an increased amount of its own or other serum-lipid in an attempt to restore full activity or perhaps accentuate the serum-sickness-producing ability of the serum. In some experiments the rabbits were injected with the serum-lipid mixed with hog-serum. By the term "lipid-free protein" we refer to serum-protein that has been treated with several lipid-solvents, e.g., alcohol, acetone or ether and has thereby been deprived of such lipid as would be removable under these conditions.

METHOD OF TREATING THE SERUM

As a general plan of procedure the serum was added drop by drop to 15 to 20 volumes of the precipitating and extracting agent. We have used absolute alcohol, 95 per cent alcohol, acetone or alcohol (abs.)-ether mixtures (1:1 or 3:1) as the precipitating

and extracting agents. The precipitated protein was recovered by filtration, again resuspended in the same or another solvent or sometimes successively in various solvents as indicated in the tables and finally dehydrated with 3 resuspensions in anhydrous ether (15 volumes each). The protein was then finally dried either in an incubator for 12 to 16 hours, or in vacuo over $CaCl_2$. Most of the lots were processed entirely at room-temperature; in some instances refrigeration was employed for original precipitation or at a later stage of the treatment. In a few instances heating at 45°C., was employed as a possible adjunct to later stages of the extractive process as will be noted in table 1. The dry powder was then added to an original volume or more of 0.9 per cent sodium chloride solution.

Re-solution occurred readily in many instances, some few preparations being slightly more turbid in appearance than the original serum. In a few instances, particularly in the case of preparations of pseudoglobulin, it was considered desirable to facilitate re-solution by the addition of small amounts of sodium hydroxide, in most of these instances acid was subsequently added in equivalent or partially equivalent amounts. All sera as finally prepared for injection were within a pH range of 6.2 to 9.2. In general it was our impression that the readiness with which re-solution occurred was in part related to the particular kind of original precipitating agent employed and placed in the order of their relative merits in this regard, we would list absolute alcohol-ether mixture 1:1; absolute alcohol-ether mixture 3:1; acetone; absolute alcohol, and 95 per cent alcohol.

A chemical preservative (usually merthiolate to give a 1:10,000 concentration) was added to many of the redissolved preparations to protect against possible growth of microörganisms. Some few lots of processed serum were filtered through a Berkefeld filter. A few were filtered through sintered glass and others were merely filtered through paper or centrifuged to remove insoluble particles and extraneous matter such as paper-lint, etc., and these were injected into rabbits without addition of preservative.

It was not apparent that ease or difficulty in securing satisfactory re-solution, or the addition of alkali, or the final H ion

concentration, or filtration or addition of preservative was in any way related to the occurrence or non-occurrence of serum-sickness.

RESULTS

In table 1 we have listed the results obtained upon injecting into rabbits the 4 lots of pseudoglobulin, 2 lots of whole serum and also various lipid-free preparations made from these sera. Under the heading of "Positive" we have listed those animals exhibiting serum-sickness on the ears, whether the reactions were marked, moderate or slight. The term "Doubtful" embraces those animals exhibiting reactions of minor nature and importance (differing, however, from any manifestations seen in the normal animals or upon the "Negative" ears) and appearing moreover during the period when serum-sickness might well be expected. These minor reactions were undoubtedly related to the injections but lacked the summation of characteristics which would permit their being classified as "positive" reactions.

It was apparent that in more than half of the 18 lipid-free preparations made from the 4 lots of pseudoglobulin, there was a significant decrease in the serum-sickness-producing quality of the sera, as observed in a lessened percentage of positive reactions. In the other preparations there was apparently partial or complete retention of the serum-sickness-producing quality. None showed an increase in this quality.

In our studies of whole sera, the experiments with serum "G" were for the most part rather satisfactory in the reduction of occurrence of serum-sickness; on the other hand in experiments with whole serum 92 there was apparently no significant reduction in the serum-sickness produced. However, serum 92 was not very active since 60 per cent of the animals receiving unaltered serum did not develop serum-sickness. One possible explanation of the difference between these 2 sera might be laid to the fact that serum 92 contained a small amount of phenol before being treated with the lipid-solvents while serum "G" contained no phenol.

With respect to the relative merits of the various procedures

TABLE 1

Serum-sickness occurring in rabbits after injection of horse-serum pseudoglobulin and lipid-free preparations (Lf) therefrom

SERUM	TREATED WITH*	PER CENT POSITIVE	PER CENT DOUBTFUL	PER CENT NEGATIVE	TOTAL NUMBER OF ANIMALS
Antidiphtheric 7936–3–4†		60	10.0	30.0	10
Lf 1	A; A	36.3		63.6	11
Lf 2	A; A	62.5	12.5	25.0	8
Lf 3	A; 95	30.0	10.0	60.0	10
Lf 5	A; A	80.0		20.0	10
Antidiphtheric 7936–5†		54.5	9.0	36.3	11
Lf 8	95; 95; A	0.0	0.0	100.0	7
Lf 9	Ac; Ac	0.0	20.0	80.0	10
Lf 10	95; A; Ac	20.0	10.0	70.0	10
Antidiphtheric 80000‡		90.0	10.0	0.0	10
Lf 4	A; 95; Ac	28.5	0.0	71.4	7
Normal 80458†		60.0	30.0	10.0	10
Lf 18	Ac; Ac	80.0	0.0	20.0	10
Lf 19	Ac; Ac; A–E 1:9	70.0	20.0	10.0	10
Lf 20	95; 95; A	30.0	40.0	30.0	10
Lf 21	95; Ac	25.0	25.0	50.0	8
Lf 25	95; E 3; 1; Ac	40.0	20.0	40.0	10
Lf 25A	95; E 3; 1; Ac	33.3	33.3	33.3	6
Lf 27	Ac; Ac; dried A–E 3:1 45° 2 hours	16.6	0.0	83.3	6
Lf 27A	Same as Lf 27	71.4	14.2	14.2	7
Lf 27B	Same as Lf 27 dissolved; Ac; Ac; dried; A–E 3:1 45° 2 hours	50.0	0.0	50.0	6
Lf 28	Ac; Ac; dried A–E 3:1 45° 2 hours	18.1	36.3	45.4	11

* In this column "A" represents absolute alcohol; "95" denotes 95 per cent alcohol; "Ac" denotes acetone; "E" denotes anhydrous ether; where figures follow A–E or 95–E they represent the proportions of the respective solvents; "45° 2 hours" implies that the dried serum-protein was heated in the alcohol-ether mixture at 45°C. for 2 hours.

In all cases the serum-proteins were dried by washing with anhydrous ether and dried in the incubator or over $CaCl_2$ before redissolving. The serum was precipitated and treated with the various lipid-solvents at room temperature (18° to 22°C.) unless otherwise stated.

† Serum furnished by Sharp & Dohme.

‡ Serum furnished by Eli Lilly & Company.

TABLE 1—*Concluded*

Serum-sickness occurring in rabbits after injection of whole horse-serum and lipid-free preparations (Lf) therefrom

SERUM	TREATED WITH*	PER CENT POSITIVE	PER CENT DOUBTFUL	PER CENT NEGATIVE	TOTAL NUMBER OF ANIMALS
"G" Normal		60.0	0.0	40.0	10
Lf 11	95; 95	30.0	10.0	60.0	10
Lf 12	Ac; Ac	50.0	10.0	40.0	10
Lf 13	95; 95; A	50.0	22.0	27.0	18
Lf 14	Ac; Ac; Ac	25.0	15.0	60.0	20
Lf 15	Ac; Ac; Ac	20.0	0.0	80.0	10
Lf 16	Ac; Ac; dried Ac 35°C. 2 hours	30.0	20.0	50.0	10
Lf 17	Ac; Ac; Ac§	20.0	10.0	70.0	10
Lf 31	A(CS); A(CS)¶**	0.0	15.3	84.6	13
Lf 32	A–E 3:1 (CS)††	23.0	53.8	23.0	13
Normal and immune antidiphtheric mixed†		15.0	25.0	60.0	20
Lf 33	A–E 1:1; Ac	9.1	27.3	63.6	11
Lf 34	A(CS) Ac	33.3	11.1	55.5	9
Lf 35	A–E 1:1; Ac	14.2	14.2	71.4	14
Lf 36	A–E 1:1; Ac	30.0	10.0	60.0	10

§ Petroleum ether used for drying in place of anhydrous ether.

¶ C.S. denotes that precipitation and extraction were carried out at 5° to 8°C.

** Treatment including precipitation with absolute alcohol, drying with anhydrous ether and dissolving the proteins, was repeated 3 times.

†† Ratio of alcohol-ether mixture was later made 1:1 by addition of ether to the mixture.

for prevention of serum-sickness, it is to be noted that whereas a certain treatment did upon occasion give a significant reduction in the amount of serum-sickness produced, the same treatment might upon another occasion with the same or other serum give a less satisfactory result. Of 3 lots of the same serum (7936–3–4) precipitated and washed with absolute alcohol, 2 (Lf 2 and Lf 5) when injected into rabbits produced serum-sickness in the usual percentage of animals, while one (Lf 1) caused serum-sickness in about half the expected number.

Equally inconsistent were the results with serum precipitated and washed with acetone and extracted with alcohol-ether mixtures at higher temperatures. In lots Lf 27, Lf 27A and Lf 27B,

the percentages of positive reactions were 16, 71 and 50 per cent respectively. In the array of technics that we have employed, it does not seem possible to look upon treatment with any particular solvent, or upon any technic involving a particular sequence of solvents as being outstanding or consistently so, for the purposeof removal of the factor causing serum-sickness.

While engaged in these studies the question arose, as to whether success or failure in elimination of the active agent might be due to complete or only partial removal of serum-lipid, which for a time focused our attention upon quantitative studies of lipid-removal by the various procedures employed. It is doubtful if any of our extractive processes completely removed the lipids since extracted serum, when later re-extracted almost invariably yielded some, though a small amount, of material to the second solvent. In 14 of the preparations the percentile removal of lipids was studied. Using the Bloor method (4), we observed in the case of preparation Lf 25 that 95 to 97 per cent of the lipid was removed through the repeated extractions therein used. This serum elicited serum-sickness in a significant proportion of the animals injected with it. In a different preparation (Lf 28) of the same serum, a removal of only 83 per cent of the lipid was observed. This latter serum, on the other hand produced fewer serum-sickness reactions than did the preparation Lf 25. In 2 preparations (Lf 25A and Lf 21) in which about 69 to 74 per cent of lipid was removed 25 to 33 per cent of rabbits showed positive reactions; with 2 preparations (Lf 27A and Lf 27) in which 74 and 92 per cent of lipid respectively had been removed, positive reactions occurred in 16 to 18 per cent of rabbits; and in one preparation (Lf 27A) in which over 94 per cent of lipid had been removed positive reactions occurred in 50 per cent of the injected rabbits. Accordingly, the opinion may be expressed that from a standpoint of quantitative relationship, lipid-removal is not necessarily correlated with removal of serum-sickness-producing factor in the serum.

Experiments were also carried out to determine whether the materials extracted from serum possessed any activity in causing serum-sickness in rabbits. For this purpose all the materials

extracted by the various solvents used with a particular lot of serum were combined; the solvents were removed by evaporation at low temperature. The residual material (lipid) was then taken up with sodium-chloride solution and mixed either with hog-serum or with the lipid-free serum from which the material had previously been extracted. These mixtures were kept at 37°C. for several hours or in cold storage over night. It was apparent that in these mixtures the lipid was not intimately com-

TABLE 2

Serum-sickness occurring in rabbits after injections of mixtures of serum-lipid and horse-serum or hog-serum

MIXTURES	PER CENT POSITIVE	PER CENT DOUBTFUL	PER CENT NEGATIVE	TOTAL NUMBER OF ANIMALS
Hog serum and lipid #1*	30.0		70	10
Hog serum and lipid #3 and #4	0	0	100	10
Hog serum and lipid #5 and #6*	0	0	100	11
Hog serum and lipid #7*	0	11.1	88.8	9
Hog serum and lipid #8*	0	0	100	5
Hog serum and lipid #9*	0	20.0	80	10
Lf serum G #11 and lipid #11*	60.0	10.0	30.0	10
Lf serum G #12 and lipid #12*	70.0	10.0	20.0	10
0.85 per cent NaCl solution and lipid #10*	0.0	0.0	100.00	10
Lf Serum G #14 and lipid #14†	0	16.6	83.3	6
Lf serum #27 and lipid #27*	0	33.3	66.6	6

* In these mixtures, the lipid was added to the serum in double the original quantity.

† The Lf serum G #14 was diluted with equal parts of sodium-chloride solution and a quantity of the lipid equivalent to that removed from twice the volume of serum ordinarily used was mixed with the diluted serum.

bined with the serum since upon standing a surface layering of lipid was usually observed.

The amount of lipid injected into a rabbit was equivalent to the lipid contained in the amount of serum ordinarily administered to animals. In some instances twice this amount was injected.

In 6 experiments the lipids (lipids 1, 3 and 4, 5 and 6, 7, 8 and 9) were combined with hog-serum before injection (table 2). This hog-serum when injected alone into rabbits in doses similar to those used in the hog-serum-lipid experiments caused serum-

sickness in only one of 17 rabbits. (We have previously shown that hog-serum is relatively inactive in this regard (5)). When the lipids were combined with hog-serum or suspended in sodium-chloride solution, no positive reactions occurred except in one experiment. In this experiment (lipid 1) the lipid combined with hog-serum was from a preparation which had shown a reduction in occurrence of reactions; however, in the experiment using lipids 3 and 4 these lipids were from preparations which had shown equal reductions in occurrence of reactions. Two other lipids combined with hog-serum (nos. 8 and 9) had been obtained from preparations which had caused no reactions when the lipid-free sera were injected and even so no reactions occurred when the lipid-extract mixtures were administered.

In the 4 experiments in which the lipids were mixed with the serum-protein preparations from which they had been removed, in 2 cases (nos. 11 and 12) the reconstituted mixtures caused the appearance of larger percentages of reactions than did the lipid-free preparations alone; in both of these lipid-free preparations, however, a significant number of positive reactions had occurred and it seemed possible that the major active agent was present in the lipid-free serum rather than in the lipid-extract. In both of the other reconstituted preparations in which lipid was added to the lipid-free serum, no positive reactions occurred, even though lipid-free sera had in both cases shown very little activity when injected alone. Activity of serum-lipid when injected alone or in combination with serum-protein seems, therefore, to be negligible in causation of serum-sickness.

The question arises as to whether the reduction in serum-sickness activity which has repeatedly been noted in these experiments may be related to a combination of circumstances, viz., a certain degree of lipid-removal associated with fundamental changes in the protein occasioned thereby. The possible denaturation of the serum-proteins, incident to treatment with lipid-solvents under these conditions, has been investigated from the standpoints of: (*a*) ability of lipid-free preparations to elicit antibody-production, for the homologous antigen as well as for untreated horse-serum; (*b*) activity of anti-horse-serum precipitin against

lipid-free preparations of serum; (*c*) ability of lipid-free serum to sensitize guinea pigs to later injections of treated and non-treated serum. In all of these respects we have not discovered any discernible evidence of denaturation of the serum-protein incident to treatment with lipid-solvents.

DISCUSSION

When horse-serum or the pseudoglobulin of horse-serum is precipitated and extracted with several lipid-solvents it appears that the percentile removal of lipids, as determined by the Bloor method, is not complete and furthermore, the amount removed by any particular method or sequence of solvents is quite irregular. Even the use of heat and alcohol-ether mixtures did not enable us to obtain constant results in relation to removal of the lipids.

These facts might serve to explain the irregularity of the results which we obtained in relation to suppression of serum-sickness when the so-called lipid-free preparations were injected into rabbits. However, in a sufficient number of experiments a removal of lipids greater than 90 per cent was obtained and even in experiments with these preparations (Lf 25, Lf 27 and Lf 27A) the reduction in occurrence of serum-sickness in the rabbits was neither satisfactory nor consistent. Furthermore, we have previously noted in quantitative studies of amounts of serum required to cause serum-sickness (1) that when pseudoglobulin is injected into rabbits a 50 per cent reduction in the amount injected, causes a marked decrease in the occurrence of reactions and with a 75 per cent reduction in the amount injected, very few reactions occur. It seems probable that in most of the lipid-free preparations a reduction in the amount of lipid of 66 per cent had taken place; therefore, had the lipid been the responsible factor in causing serum-sickness, the suppression of reactions should have been constant and far greater.

In addition the injection into rabbits of the materials extracted from the serum by the lipid-solvents, did not with any regularity or to any appreciable degree effect the occurrence of serum-sickness. In fact the lipid-extracts appeared to be quite inert in

this regard. It is, of course, possible that either the lipids were altered in such a manner during the handling and before they were recombined with serum-proteins so that they no longer retained their original activity or that the lipid-extracts did not combine with the serum-proteins in such a manner that they could serve to cause serum-sickness.

Taking both the evidence resulting from injections of the lipid-free preparations and the combinations of lipid-extracts with serum it does not appear that serum-lipids play any part in causing serum-sickness in rabbits.

The rather frequent decrease of incidence of serum-sickness in rabbits injected with the various lipid-free preparations is not easily explained. Our previous and later experience with various lots of sera does not justify the assumption that the percentile variations noted in the experiments reported here are of such nature as one might expect in different lots of rabbits injected at different times with the same sample of serum. This is particularly true if one considers only the percentage of negative animals; there will occur a slightly different distribution of positive reactions and doubtful reactions but the number of negative animals remains within certain limits fairly constant. It appears that the results cannot be explained upon the basis of the experimental variation in reaction-capacity of different lots of animals. Neither is it possible to explain the diminution in serum-sickness noted in some experiments on the basis of denaturation of the serum-protein. The serum-protein is apparently as active antigenically as the untreated serum-protein and could not by either precipitative tests or by anaphylactic reactions be differentiated from the normal serum-protein. It is possible, of course, that the protein was altered by the treatment with lipid-solvents in a manner not detectable by the usual immunologic methods.

CONCLUSIONS

The treatment of horse-serum or pseudoglobulin with lipid-solvents, in the manner which we have described above, may reduce the activity of the serum in causing serum-sickness, but such treatment of serum cannot be depended upon invariably to

remove or destroy the factor concerned in causing serum-sickness in rabbits.

The loss of the serum-sickness-producing factor is not directly correlated with quantitative removal of lipid.

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