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Effect of Neonatal Injections of Protein on the Immune Response to Protein-Hapten Complexes

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Summary. Thirteen out of fourteen normal mature rabbits injected with sulphanil-azo-human-serum-albumin produced antibodies against sulphanilic acid.

In contrast, out of fifteen mature rabbits which had received human serum albumin at birth, none produced detectable antibodies to sulphanilic acid after injections of the sulphanilazo-human-serum-albumin.

Fourteen out of fifteen normal mature rabbits produced antibodies to sulphanilic acid after injections of sulphanilazo-rabbit-serum-albumin.

One out of nine mature rabbits which had been injected neonatally with sulphanilic acid produced antibodies against the hapten when immunized with the sulphanilazo-human-serum-albumin.

The implications of these results are discussed in relation to some current views on antibody formation.

INTRODUCTION

The investigation reported in this paper represents a combination of two rather different approaches to the problem of the mechanism of antibody production. The first of these approaches was developed mainly by Landsteiner and his colleagues (see Landsteiner, 1947), after the demonstration that the injection of animals with small amounts of relatively simple chemical compounds, such as arsanilic acid, linked chemically to a protein antigen, resulted in the formation of antibodies reactive with the chemical compound or hapten. Investigations with many chemically different haptenic groups revealed the amazing specificity of the immune response and indicated the essential role of the protein in the elicitation of antibody production against a small determinant group. However, the reason why a carrier macromolecule is necessary is unknown.

The second approach is based on the prediction by Burnet and Fenner (1949) that contact of the tissues of an embryo with a given antigen will render the animal, when mature, incapable of responding immunologically to that antigen. The correctness of the prediction has been amply demonstrated in the case of living homologous cells by Medawar and others (see review by Brent and Medawar, 1959), while others have shown that specific immunological unresponsiveness can also be produced by injections of certain soluble heterologous protein antigens before, or soon after, birth (Hanan and Oyama, 1954; Cinader and Dubert, 1955; Dixon and Maurer, 1955; Smith and Bridges, 1958). Experiments along these lines have acted as a great stimulus to immunological thought. So far, however, this approach has not provided sufficient information to help us choose

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definitely between the ever-increasing number of theories of antibody production. Moreover, it is not even certain that artificially induced immune unresponsiveness, particularly that resulting from neonatal injections of soluble antigens, is due to the same mechanisms as is the natural unresponsiveness of an individual animal to its own proteins.

Experiments involving a combination of both these approaches were carried out previously by Cinader and his colleagues (Cinader and Dubert, 1955; Cinader and Pearce, 1958), who used simple chemical compounds such as sulphanilic acid as haptens to study the specificity of immunological tolerance induced by injections of antigen into neonatal rabbits. Certain of the experimental groups in the work described in the present paper correspond closely to some of Cinader's groups. The results, although similar to Cinader's, are not identical. The possible causes of the differences will be discussed below.

In the present study rabbits were injected at birth with human serum albumin (HSA). Later these rabbits were tested and compared with normal rabbits for their capacity to produce antibodies against HSA, and against sulphanilic acid linked to HSA, to bovine serum albumin (BSA) and to rabbit serum albumin (RSA). Some animals were injected at birth with sulphanilic acid alone: later the response in these and in normal rabbits was measured after injections of sulphanilic acid bound to HSA or to BSA. The response of normal adult rabbits to the injection of their own serum proteins linked with sulphanilic acid was also tested.

The response of the animals within the various groups was surprisingly uniform, and clear-cut differences were seen between certain of the groups. The implications of these differences will be discussed in relation both to current theories of antibody formation and to current notions on the mechanisms of immunological tolerance.

MATERIAL AND METHODS

ANTIGENS. Human serum albumin (HSA), bovine serum albumin (BSA) and rabbit serum albumin (RSA) were obtained from the Biochemical Department, Statens Serum-institut.

SULPHANILAZO-PROTEINS were prepared by coupling one part diazotized sulphanilic acid with ten parts of the various proteins at pH 8.0 for 1 hour, followed by extensive dialysis against saline in the cold for 2 days (Kabat and Mayer, 1948).

ANIMALS. Newborn rabbits (both sexes) and adult male rabbits (2000–3000 g.) were used in the experiments.

SEROLOGY. Sera were titrated for antibodies in the tannic acid haemagglutination test. The details of the technique were as previously described by Boyden and Sorkin (1955). Twofold dilutions of the test sera were made starting at a concentration of 1/10.

RESULTS

IMMUNE RESPONSES OF MATURE RABBITS INJECTED NEONATALLY WITH HSA

Two similar experiments (1a and 1b), which differed only in small details, are described below.

In both experiments rabbits were injected within 24 hours of birth with 100 mg. HSA. Further injections of 10 mg. HSA were subsequently given at intervals of several weeks.

When the animals were 4–5 months old, they were divided into three groups. The groups received five intravenous injections, spaced over about 2 weeks, of sulphanilazo-HSA, sulphanilazo-RSA and sulphanilazo-BSA respectively. Three groups of normal rabbits (same age and weight and same sex distribution) were similarly injected. Blood was taken for serum 10 days after the last injection.

The sera were tested in the tannic acid haemagglutination test for antibodies to sulphanilic acid (using red cells coated with sulphanilazo-RSA), to HSA and to RSA.

The details of the experiments were as follows:

Experiment 1a

Day 1: Newborn rabbits injected within 24 hours of birth with 100 mg. HSA in 1 ml. saline intraperitoneally. *Days 3, 8, 73 and 96*: Same rabbits injected with 10 mg. HSA in 1 ml. saline subcutaneously. *Day 81*: 10 ml. blood taken from all rabbits. Test sera for antibodies to HSA (all were negative). *Day 171*: Fifteen of the rabbits injected on day 1 were divided into three groups (1, 2, 3), five in each group. The sexes were about equally distributed among the groups. Fifteen *normal* rabbits of the same age and stock constituted *groups* 4, 5 *and* 6.

Injections were made as follows:

Groups 1 and 4: 1 ml. sulphanilazo-HSA 2.5 mg./ml. intravenously.
Groups 2 and 5: 1 ml. sulphanilazo-RSA 2.5 mg./ml. intravenously.
Groups 3 and 6: 1 ml. sulphanilazo-BSA 2.5 mg./ml. intravenously.

Days 175, 179, 182, and 185: Injections as on day 171. *Day 193*: 10 ml. blood taken from all rabbits for serum.

Experiment 1b

Days 1 and 2: Newborn rabbits injected within 24 hours of birth with 100 mg. HSA in 1 ml. saline intraperitoneally. *Day 90*: All these rabbits injected with 10 mg. HSA in 1 ml. saline intravenously. *Day 105*: 10 ml. blood taken from all rabbits. Test sera for antibodies to HSA (all were negative). *Day 172*: Thirty of the rabbits injected on day 1 were divided into three groups (1, 2, 3), ten in each group. The sexes were about evenly distributed among the groups. Thirty *normal* rabbits of the same age and stock constituted *groups* 4, 5 *and* 6.

Injections were made as follows:

Groups 1 and 4: 1 ml. sulphanilazo-HSA 2.5 mg./ml. intravenously.
Groups 2 and 5: 1 ml. sulphanilazo-RSA 2.5 mg./ml. intravenously.
Groups 3 and 6: 1 ml. sulphanilazo-BSA 2.5 mg./ml. intravenously.

Days 176, 180, 183 and 185: Injections as on day 172. *Day 192*: Bleed all 10 ml.

The primary interest in these experiments lies in the immune response to the sulphanilic acid group. The titres of the sera of the individual rabbit for antibodies against sulphanilic acid are given in Table 1 (in which the results from experiments 1*a* and 1*b* are combined).

TABLE 1

RESPONSES OF MATURE RABBITS INJECTED NEONATALLY WITH HSA

Group	*Injection at birth*	*Injection at 5–6 months*	*Antibody titres to sulphanilic acid*
1	HSA	Sulphanilazo-HSA	0. 0. 0. 0. 0. 0. 0. 0. 0. 0. 0. 0. 0. 0. 0.
2	HSA	Sulphanilazo-RSA	0. 7. 4. 8. 8. 6. 5. 5. 5. 3. 5. 6. 8. 3. 5.
3	HSA	Sulphanilazo-BSA	9. 6. 7. 6. 10. 5. 7. 0. 7. 3. 2. 6. 4. 0. 4.
4	Nothing	Sulphanilazo-HSA	6. 9. 8. 6. 2. 5. 1. 5. 8. 1. 2. 0. 2. 4.
5	Nothing	Sulphanilazo-RSA	4. 4. 4. 1. 8. 8. 11. 11. 3. 10. 6. 9. 9. 11. 8.
6	Nothing	Sulphanilazo-BSA	12. 11. 7. 7. 7. 9. 4. 10. 7. 8. 8. 8. 1. 11. 8.

Note: Each figure represents the haemagglutination titre of one serum. It refers to the last tube in the row of serial twofold dilutions showing a positive haemagglutination pattern.

Thus 0 = no reaction at 1/10
so that 1 = 1/10 dilution
2 = 1/20 dilution
3 = 1/40 dilution, and so on.

The results may be summarized as follows:

(1) of fifteen rabbits injected at birth with HSA and later with sulphanilazo-HSA, none produced detectable antibodies against sulphanilic acid (group 1).

In contrast, of fourteen rabbits not injected with HSA at birth, thirteen responded to injections of sulphanilazo-HSA with the production of detectable antibodies against sulphanilic acid (group 4).

(2) of fifteen rabbits injected at birth with HSA and later with sulphanilazo-RSA, fourteen produced detectable antibodies against sulphanilic acid (group 2).

Of fifteen rabbits not injected with HSA at birth, fifteen responded to injections of sulphanilazo-RSA with the production of detectable antibodies against sulphanilic acid (group 5).

(3) Of fifteen rabbits injected at birth with HSA and later with sulphanilazo-BSA, thirteen produced antibodies against sulphanilic acid (group 3).

Of fifteen rabbits not injected with HSA at birth, fifteen responded to injections of sulphanilazo-BSA with the production of detectable antibodies against sulphanilic acid (group 6).

The sera were also tested for antibodies against HSA. Some weak reactions were obtained in group 4 (injected with sulphanilazo-HSA as adults, but not injected at birth). Other sera were negative.

RESPONSE OF NORMAL RABBITS TO THE INJECTION OF AUTOLOGOUS SERUM PROTEINS LINKED WITH SULPHANILIC ACID

In the experiments described above, it was found that animals rendered unresponsive to HSA did not produce detectable antibodies to sulphanilic acid when injected with sulphanilazo-HSA. On the other hand, when rabbits were injected with the hapten coupled to RSA, to which they were presumably tolerant by nature, there was a very good antibody response to the hapten. The somewhat remote possibility was considered that this discrepancy might be due to the presence in the RSA preparation of homologous proteins which differed antigenically from the autologous proteins of the host, and that the immune response was invoked by complexes of the hapten with these homologous proteins to which the animal was not immunologically tolerant. Another experiment was therefore set up in which rabbits were immunized with autologous serum proteins coupled with sulphanilic acid. (The dose of antigen, injection schedules, etc., were as in experiments 1a and 1b.) The sera of these rabbits were found to contain antibodies to sulphanilic acid, the titres being similar to those obtained in groups 2 and 5 in experiments 1a and 1b.

RESPONSES OF MATURE RABBITS INJECTED NEONATALLY WITH SODIUM SULPHANILATE

In the experiment described in detail below newborn rabbits were injected at birth with 10 mg. sodium sulphanilate. One and a half and 3 months later they were reinjected with 10 mg. sodium sulphanilate.

When $4\frac{1}{2}$ months old the animals which had received sodium sulphanilate at birth were divided into two groups, one of which was injected with sulphanilazo-HSA, while the other received no injection. A group of normal rabbits (not injected at birth) also received injections of sulphanilazo-HSA.

Experiment 2

Days 1 to 3: Newborn rabbits injected with 10 mg. sodium sulphanilate in 1 ml. saline intraperitoneally. *Day 46*: Same rabbits injected with 10 mg. sodium sulphanilate in 1 ml. saline subcutaneously. *Day 92*: Injections as on day 46. *Day 140*: 10 ml. blood taken from all rabbits. *Day 141*: Rabbits were arranged in three groups and injected as follows:

Group 1: Nine rabbits (injected with sodium sulphanilate at birth). Injected with 10 mg. sulphanilazo-HSA.

Group 2: Eight rabbits (injected with sodium sulphanilate at birth). No injection.

Group 3: Ten normal rabbits (same age as groups 1 and 2). Injected with 10 mg. sulphanilazo-HSA.

All the injections were made in 1 ml. saline intravenously.

Days 145, 149, 152 and 156: Injected as on day 141. *Day 165*: 10 ml. taken from all rabbits for sera. The sera were tested in the tannic acid haemagglutination test for antibodies against sulphanilic acid and HSA.

The results of this experiment are shown in Table 2.

All the nine rabbits injected neonatally with sulphanilic acid, and later injected with sulphanilazo-HSA, produced antibodies to sulphanilic acid (group 1). Similarly, sera of all of the ten normal rabbits immunized with sulphanilazo-HSA contained antibodies to the hapten (group 3). As expected, no antibodies were detected in the sera of animals which had received sulphanilic acid only (group 2).

TABLE 2

RESPONSES OF MATURE RABBITS INJECTED NEONATALLY WITH HAPTEN

Group	*Injection at birth*	*Injection later*	*Antibody titres to sulphanilic acid*
1	Sulphanilic acid	Sulphanilazo-HSA	4. 4. 3. 6. 3. 6. 6. 6. 5.
2	Sulphanilic acid	Nothing	0. 0. 0. 0. 0. 0. 0. 0.
3	Nothing	Sulphanilazo-HSA	4. 5. 3. 1. 2. 1. 2. 7. 8. 8.

Note: Each figure represents the haemagglutination titre of one serum. It refers to the last tube in the row of serial twofold dilutions showing a positive haemagglutination pattern
Thus 0 = no reaction at 1/10
so that 1 = 1/10 dilution
2 = 1/20 dilution
3 = 1/40 dilution, and so on.

A similar experiment to this was carried out with twelve animals in each group. The results of the second experiment were in complete agreement with those of the first described above. Thus, under the conditions of these experiments, the injection of hapten alone at birth did not induce a state of immune unresponsiveness towards the same hapten injected later in life coupled to a protein.

DISCUSSION

The implications of the results described above will be discussed under three headings.

1. HSA AT BIRTH, HSA-HAPTEN: NO ANTIBODIES FORMED TO HSA OR HAPTEN

This result seems to indicate that when immune unresponsiveness is induced by injection at birth of a given foreign protein, later injections of the mature animal with the same protein linked to a new haptenic group cause no antibody formation either to the protein or to the hapten.

There is some discrepancy between this result and the findings of Cinader *et al.* (Cinader and Dubert, 1955; Cinader and Pearce, 1958), who also injected HSA into eight rabbits at birth and later injected them with sulphanilazo-HSA. Three of these rabbits produced antibodies against the haptenic group. (All their control rabbits, not injected with HSA at birth but injected as adults with sulphanilazo-HSA were able to produce antibodies to the haptenic group.) One possible explanation of this discrepancy is that the HSA preparation used by Cinader *et al.* might have contained proteins other than HSA in amounts which were too small to induce tolerance at neonatal injection but which, when coupled to hapten, gave rise to an immune response in three of the eight mature animals.

The lack of response to hapten coupled with HSA in all fifteen animals unresponsive to HSA is somewhat difficult to reconcile with the clonal selection theory of antibody production in its present form (Burnet, 1959; Lederberg, 1959), since according to this theory, immune unresponsiveness against a given determinant group is the consequence

of the elimination, during an immature phase, of all lymphoid cells which are genetically endowed with the capacity to make antibodies against the determinant group in question. In terms of the clonal selection theory, the injection of HSA at birth would result in the elimination of potential antibody producers against the determinant groups of HSA, but clones capable of eventually producing antibody against the hapten should not be affected. Moreover, the finding that animals unresponsive to HSA do not respond immunologically to a hapten linked to HSA suggests, although it does not prove, that in a normal animal the immune response to the hapten part of a hapten-protein complex is due to the same cells as is the response to the protein part of the complex. This suggestion is also inconsistent with the clonal selection theory.

In an animal rendered unresponsive to HSA, the lack of response to a hapten linked to HSA can hardly be due to the neutralization or destruction of cell receptors for the hapten group (Ehrlich, 1900; Boyden, 1960), of natural antibodies which happen to possess an affinity for the hapten (Jerne, 1955) or of cells specifically reactive to the haptenic group (Burnet, 1959; Lederberg, 1959). It seems that the lack of response to the hapten can best be explained as being due to some active process specifically directed against the HSA part of the molecule, and that this active process is the consequence of a specific response of the neonatal tissues to the antigen.

The experiments provide no clue as to the nature of this specific neonatal response. We can conclude only that the response in some way interferes with the capacity of the animal to respond to the antigen when exposed to it in later life.

At first consideration it might seem reasonable to suggest that some circulating antibody-like factor, not detectable by ordinary immunological tests, is produced which reacts with the antigen, preventing the entry of the latter into the antibody-producing cells when they mature. There are two reasons for discounting this view:

(i) The rate of elimination of antigen from the blood stream in an unresponsive animal is the same as in the normal animal (Smith and Bridges, 1958). This speaks against the existence in the serum of the unresponsive animal of any specific factor which combines with the antigen in the blood stream.

(ii) If immune unresponsiveness were due to a serum factor which reacted specifically with injected antigen to prevent its uptake by antibody-producing cells, then it should be possible to induce an antibody response in such unresponsive animals merely by injecting antigen in excess of this factor. The evidence indicates that it is not possible to break immune unresponsiveness by injecting excess antigen.

An explanation which, although it stretches the imagination somewhat, seems to fit the facts, is as follows: The injection of HSA at birth elicits in the rabbit a response, the effect of which is to specifically interfere with the production of antibodies against this antigen, perhaps by inhibiting or destroying by a 'homograft-like' reaction any cells which take up the antigen or which commence to make antibodies against it. Conceivably this response might involve the production of a host substance carrying specific groupings analogous in structure to those of the antigen, instead of complementary to them as in the case of antibodies. Thus, when an animal unresponsive to a given protein is injected with this protein linked to a hapten, the potential antibody-producing cells which take up the complex will be specifically eliminated (or inhibited), so that no antibodies would appear either to the protein or to the hapten.

2. THE INJECTION OF RSA-HAPTEN (OR HAPTEN LINKED TO AUTOLOGOUS SERUM PROTEIN) INTO NORMAL RABBITS: ANTIBODIES ARE PRODUCED AGAINST THE HAPTEN

This result is not surprising in view of much evidence which suggests that if a small haptenic group becomes linked chemically to an autologous protein, an immune response will occur (see Chase, 1954; Eisen, 1959).

The main implication of this result, when considered in relation to the finding that HSA-hapten causes no immune response in animals unresponsive to HSA, is that unresponsiveness induced by the injection of an antigen at birth is basically different in mechanism from natural unresponsiveness against indigenous or 'self' proteins. It might be argued against this conclusion that the denaturation involved in the attachment of the hapten to the protein molecule changes the latter so much that it is no longer regarded as a self-component. However, this argument is not valid, since the same would apply to the HSA system in which the unresponsiveness is induced by the HSA molecule before any treatment with hapten.

3. HAPTEN AT BIRTH, HSA-HAPTEN LATER: ANTIBODIES ARE PRODUCED TO HAPTEN AND TO HSA

This result indicates that the unresponsive state, like antibody production itself, requires for its induction the participation, as part of the antigenic complex, of a macromolecule. Sulphanilic acid alone can neither give rise to antibody formation in the adult nor can it induce immune unresponsiveness when injected neonatally.

This finding is consistent with the view suggested above that the injection of antigens into neonatal animals induces some sort of specific response, perhaps an alternative to antibody formation, which in later life specifically interferes with the animal's capacity to produce antibodies against the antigen in question.

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