not certain whether changes in such qualities as structures or habits, assortative matings or interracial sterilities come first. No one knows whether a group of animals by living in a new habitat gradually acquires new structures or whether animals with peculiar structures are especially fitted from the beginning to live in different habitats than those occupied by the parent stock.

Available knowledge shows that there has been evolution. The mechanisms of heredity are quite well known. The great biological mystery to-day is variation. About all that scientists at the present time are able to do is point out the conditions under which animals vary. So there are well-understood examples of hybridization, the establishment of pure lines, the changing of the phylogenetic record as represented during ontogeny by acceleration and larval adaptation, even the modification of gene characters by experimentally controlled environmental factors; but why animals vary no one knows.

When factors which have played a leading rôle in the formation of species are considered, an ecologist thinks first of environment. Present-day evidence does not indicate that environment has caused animals to vary. It does suggest that new species have arisen by segregation—structural, physiological, reproductive, genetic, habitatic and biographical. Ecological segregation is one factor which has been associated with the production of new species, which are perhaps at times produced by competitive, struggling selection and at times by groups of animals which by becoming adapted to peculiar and previously unoccupied niches in environment are able to escape competition. *Quien sabe?*

TISSUE REACTIONS IN IMMUNITY: XIV. THE SPECIFIC REACTING CAPACITIES OF DIFFERENT TISSUES OF AN IMMUNIZED ANIMAL[1,2]

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THE protective forces of a bacterial-immunized animal, according to modern immunologic knowledge, are centered largely in the fluids and the wandering cells. The great controversy of half a century ago regarding the humoral and cellular theories of immunity is at present of historical interest only; it is now generally accepted that humoral antibodies as well as phagocytes are lined up in defense of the host when attacked by bacteria. Although bacterial attacks generally involve fixed tissues, such as the skin, muscle, etc., yet the rôle of these tissues in immunity is far from established. Indeed, these tissues are regarded as hypersensitive to the very organisms against which the protective forces of the fluids and phagocytes are directed.

Let us consider two basic and readily measurable responses of a rabbit immunized with a protein solution or a bacterial suspension, namely, the reaction between serum and antigen and the reaction between skin and antigen. The former is classed under the familiar antigen-antibody reactions, while the latter is referred to as local anaphylaxis, tissue hypersusceptibility or hypersensitiveness. The basis for the latter terminology is the fact that specific antigen injected into the skin of an immunized rabbit calls forth an inflammatory response not given by a normal rabbit. It is this inflammatory response that is interpreted to be the result of a specific hypersusceptible state of the skin.

In spite of this interpretation of skin hypersusceptibility to the antigen, it is not generally assumed that the skin actually enters into a union with the antigen in producing the inflammatory reaction. The view is prevalent that the inflammation is due to an interaction between circulating antibodies and the introduced antigen, that this interaction results in substances toxic to the tissues, thus causing the inflammatory response. If this view is accepted, it would appear that the tissue in which the specific inflammation occurs is merely a "neutral bystander" in a reaction that takes place between an antigen and antibody.

The experimental data to be herewith considered

[1] This paper contains an account of the work by Dr. Kahn for which the eleventh annual award of the American Association for the Advancement of Science was made at the Boston meeting.

[2] For previous publications in this field, the reader is referred to: R. L. Kahn, "Skin Response as a Measure of Immunization and Sensitization," *Jour. Bacter.*, 25: 81, January, 1933; "Studies on Sensitization," Papers I to VI, *Proceed. Soc. Exp. Biol. and Med.*, 30: 603, March, 1933; "Studies on Tissue Reactions in Immunity," Papers VII to XIII, *Jour. Immunol.*, 25: 295, October, 1933. Papers XV and XVI, in press, give detailed experimental data of the present article.

question the fitness of the terms "local anaphylaxis" or "tissue hypersensitiveness" when applied to tissue-antigen reactions in an immunized rabbit, and do not confirm the view that these reactions are the result of the union between antigen and circulating antibodies. The data indicate that tissue-antigen reactions belong in the same class with serum-antigen reactions; that both reactions are manifestations of a property acquired in common by the tissues, fixed as well as fluid, as a result of immunization; namely, the property to combine with antigen. The data further indicate that this capacity to combine with antigen differs quantitatively with different tissues, some possessing the capacity to a greater degree than others.

EXPERIMENTAL

Specific Tissue Reactions of an Immunized Animal are Independent of Serum Antibodies

We shall first attempt to answer the question as to whether or not the tissue-antigen reaction of a protein-immunized animal is dependent upon circulating antibodies. Elsewhere we have described a simple method whereby a comparison of the capacities of the skin and of the serum to react with antigen can be established in a protein-immunized rabbit by determining simultaneously the skin-reacting and serum-precipitin titers. The method is carried out as follows: An albino rabbit is immunized by means of one or more injections of a protein solution, such as human serum. At the time set for determining the precipitin and skin reactions, a series of dilutions of this serum with physiologic salt solution is prepared so as to have undiluted serum and a 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000 dilution. For precipitin tests, 0.1 cc quantities of these dilutions are mixed with 0.1 cc amounts of undiluted immune rabbit serum and the mixtures incubated for 1 hour at 37° C., followed by about 18 hours at icebox temperature. The highest dilution of human serum giving a precipitate with the rabbit serum is considered the precipitin titer. For skin tests, 0.1 cc quantities of the same dilutions of human serum are injected into the hair-clipped skin of the immune rabbit. The injections are given about 2 inches apart on one side of the animal and the reactions are noted after 24 hours. The highest dilution of the human serum producing an inflammatory response is considered as the skin-reacting titer.

If a rabbit is immunized with a very small amount of human serum such as one injection of 0.1 cc of a 1:10 dilution with physiologic salt solution, it will be found that, in about two weeks, the serum-precipitin titer may reach 1,000, while the skin-reacting titer may be somewhat lower. In the course of a month, however, the serum-precipitin titer will probably become negative, while the skin-reacting titer may be as high as 1,000, *i.e.*, 0.1 cc of 1:1,000 dilution of serum causing the production of an inflammatory response. With immunizing injections of larger quantities of serum, the presence of the precipitins will be prolonged, but on their disappearance, the skin-reacting titer has invariably been found to be relatively high. The result of this experiment indicates that the skin-antigen reaction is not dependent upon serum precipitins, also that the skin reaction is a more permanent response than the serum reaction.

Let us now turn to an experiment wherein skin and serum reactions were simultaneously determined in bacterial-immunized rabbits. Two groups of rabbits were chosen. One group was immunized by repeated injections of killed typhoid suspensions, given intracutaneously, and the second group by similar injections administered intravenously. From time to time the serum-agglutinin and skin-reacting titers were determined simultaneously. The former titer was established in the usual way. In determining the skin titer, a series of bacterial suspensions was prepared, in multiples of 10, ranging from 1,000,000,000 to 100,000 or 10,000 organisms per cubic centimeter, and intracutaneous injections of 0.1 cc quantities of these suspensions were given as in the case of the serum dilutions in the previous experiments. The least number of organisms in a suspension producing an inflammatory response in the skin 24 hours after the injection represented the skin-reacting titer to the organisms.

Judging from the results of this experiment, no direct relation exists between the serum-agglutinin titer and skin-reacting titer to the bacterial suspension. It was found that the rabbits immunized by cutaneous injection showed a high skin titer and a low agglutinin titer, while the rabbits immunized by intravenous injection showed a high agglutinin titer and a low skin titer. If the skin reactions were dependent upon serum agglutinins the two types of titers would tend to parallel one another. It would appear, therefore, that so far as the skin reaction is concerned, the bacterial-immunized rabbit does not differ from the protein-immunized animal; that in both cases the tissue reaction is independent of serum antibodies.

Tissues of an Animal Undergo a Definite Change as a Result of Immunization

Thus far, the experiments cited have demonstrated changes in the skin of rabbits as a result of immunization. The following experiment will demonstrate similar changes in other tissues. The experimental plan utilizes horse serum as the immunizing agent and diphtheria toxin with its specific horse-serum antitoxin as the agents for establishing that the

various tissues of the rabbit have undergone a definite change as a result of the immunization with horse serum. Thus, if a given dose of diphtheria toxin, such as 50 MLD, is injected intracutaneously in a normal rabbit and simultaneously a liberal dose of horse-serum antitoxin, such as 50 units, is injected in another area in the skin, the animal will show little effect from the injected toxin. If, however, the same quantities of toxin and antitoxin are injected under the same conditions in a rabbit immune to horse serum, the animal will be found to succumb to the toxin. The antitoxin, being part of horse serum, apparently enters into some union with the skin and is thereby prevented from diffusing into the tissues where it could meet and neutralize the toxin.

Let us consider a more comprehensive experiment. Six groups of horse-serum-immunized rabbits were injected intracutaneously with 50 MLD toxin. Each animal was then immediately injected with 50 units of antitoxin—group 1, intracutaneously; group 2, subcutaneously; group 3, intraperitoneally; group 4, intravenously; group 5, intramuscularly; and group 6, intracerebrally. All the animals, without exception, succumbed to the toxin. Non-immunized as well as human-serum-immunized control rabbits that received similar toxin and antitoxin injections survived without exception.

It is evident from the results of this experiment that different tissues, fixed as well as fluid, of a protein-immunized rabbit undergo a definite change as a result of immunization. This change apparently is of such character as to cause these tissues to combine with the injected antigen and prevent its diffusion into the surrounding tissues.

Tissues of an Immunized Animal Possess Different Capacities for Reacting with Antigen

The problem arose as to whether it might be possible to measure in horse-serum-immunized rabbits the extent of the union of different tissues with horse-serum antitoxin. If this were possible, we would have a measure of the antigen-combining capacity of the different tissues of an immunized animal, which in turn would indicate the extent of the immunity of the different tissues. The following approach suggested itself: to inject a standard dose of diphtheria toxin, such as 50 MLD, in a group of rabbits similarly immunized with horse serum and to determine the number of units of antitoxin, when injected by different routes, that will save these animals from toxin death. If, for example, they should be saved by the same number of units, whether injected intracutaneously or intramuscularly, one would assume that these two tissues have the same combining capacity for antitoxin. If, however, a widely different number of units would be required to save the rabbits, depending on the route of injection, then it would be necessary to assume that these two tissues possess different combining capacities for the antitoxin.

An experiment of this nature was carried out wherein the antitoxin was injected not only intracutaneously in one group and intramuscularly in another, but also subcutaneously, intraperitoneally, intravenously and intracranially in four corresponding groups. It was found that in order to save these horse-serum-immunized rabbits from death from the 50 MLD toxin: 1,500 units of antitoxin were necessary, when injected intracutaneously; 1,000 units, when injected either subcutaneously or intraperitoneally; 100 units, when injected intramuscularly or intracerebrally; and 75 units, when injected intravenously. The administration of less antitoxin, such as 1,000 units intracutaneously; 700 units subcutaneously; 750 intraperitoneally; 75 units intramuscularly or intracerebrally, and 50 units intravenously, did not prevent death of the rabbits from the toxin. In the case of the control non-immunized rabbits, it was found that in order to save these animals from toxin death: 20 units of antitoxin were necessary when injected intracutaneously or subcutaneously; 7.5 units, when injected intraperitoneally; 10 units, when injected intramuscularly or intracerebrally; and 5 units, when injected intraveneously.

It is evident from these results that the skin and the peritoneal tissues of horse-serum-immunized rabbits possess a capacity for reacting with horse-serum antitoxin that is approximately ten times as great as that of *in vivo* plasma, skeletal muscle or brain tissue. Stated more generally, the reacting capacity for specific antigen, or the degree of immunity, of skin and peritoneal tissues of a protein-immunized rabbit is approximately ten times as great as that of the other tissues studied. It is also clear that the skin of non-immunized rabbits possesses a non-specific fixation capacity for antitoxin greater than that possessed by the other tissues. It should be added that in these toxin and antitoxin experiments, the immunization of the rabbits with horse serum was carried out by means of an initial intravenous injection of 0.2 cc serum per kg of body weight followed by, in 10 days, a second injection of 0.1 cc per kg, given either subcutaneously or intravenously. The toxin and antitoxin injections were made 6 to 8 days after the second immunizing injection. Another method of immunization of rabbits with horse serum would undoubtedly quantitatively affect the antigen-combining capacities of the tissues investigated.

DISCUSSION

One should necessarily be cautious when discussing the significance of data which tend to upset accepted views in a field of science. Such views are often the result of cumulative scientific thought and should be questioned only after unshakable experimental proof. Yet one can not escape the conclusion from the experiments summarized in this article that immunization with a protein is not first and foremost a matter of the production of circulating antibodies; nor is it a condition wherein the reactions between serum and antigen are manifestations of immunity, while the reactions between tissue and antigen are manifestations of susceptibility. The studies herewith recorded indicate that the tissues of an animal, whether they be fluid or fixed, acquire a characteristic property as a result of immunization, namely, the capacity to detect and enter into combination with the antigen whenever they come in contact with it. If we were to take a bacterial-immunized animal as an illustration, we would consider that the reactions between serum and antigen, phagocytes and antigen, and skin or muscle and antigen, represent manifestations of the same basic response, due to a change that has taken place in the tissues as a result of immunization. These three reactions, incidentally, undoubtedly have the same immunologic function, namely, destruction of the parasite.

One is also forced to conclude from these experiments that the immunologic response of the skin, as measured by its capacity to combine with antigen, is far greater than that of *in vivo* plasma. This fact may help explain why skin tests in bacterial infections are more delicate than serum-agglutinin tests, as, for example, in *Brucella abortus* infections. Another illustration is the immunity of human skin to the staphylococci, as exemplified by the fact that, in practically all cases, whenever these organisms gain a foothold in this tissue they are prevented from entering into the deeper tissues by local destruction and outward elimination, although the serum shows no indications of the presence of agglutinins against them. It is perhaps to be expected that the immunity of the skin would be high, considering that this tissue through the ages has been the most exposed to parasitic attack. With regard to the high immunologic response of the peritoneal tissue, it is known that the cellular elements in the peritoneal cavity are apparently always in an active state.[3] It is possible that this high activity is at least in part responsible for their high antigen-reacting capacity.

Immunity is an extremely complex phenomenon and embraces a chain of responses, many of which are not identical in different animals even when the same organism is the infecting agent. The findings reported in this article apply to the rabbit, but it is believed that in a measure they apply also to man. Both the rabbit and man produce local skin reactions to antigen and show a tendency for ready antibody production as a result of immunization, and both are not susceptible to anaphylactic shock to the same extent as, for example, the guinea pig. In all probability, therefore, in man, as in the rabbit, the primary immunologic response is unitarian in nature and consists of a newly acquired capacity of all the tissues to combine with antigen; also that this capacity of the cutaneous and peritoneal tissues is many times greater than that of the skeletal muscle, brain and *in vivo* plasma.

The fate of antigen after it is injected into the tissues of an immunized rabbit is now being investigated in this laboratory by means of toxin and antitoxin experiments. Preliminary studies indicate that the union of the tissues of a horse-serum-immunized rabbit with horse-serum antitoxin is not passive but active, the latter being actually modified or destroyed in the reaction. If, for example, horse-serum antitoxin is injected into the skin of a horse-serum-immunized rabbit and, after 24 to 48 hours, a limited dose of toxin is injected into the same area, no neutralizing action is noted, indicating loss of antitoxic powers. Another problem under investigation is the extent of the antigen-combining capacity of tissues other than those studied, such as the smooth muscle tissue, lymph glands, visceral organs, etc. It is obviously of importance to investigate as many tissues as possible and thus obtain a quantitative measure of the extent of their immunologic response. It is indeed reasonable to assume that bacterial infection will be more successfully combated when the degree of immunity of all the tissues of an immunized animal has been definitely established. Studies are also being directed to the question whether in toxin-immunized rabbits, different tissues might possess different toxin-neutralizing capacities, independently of the blood plasma.

CONCLUSIONS

A method is described which makes possible the measurement of the degree of immunity of different tissues in a protein-immunized rabbit by quantitatively establishing the capacities of the tissues to react with specific antigen. By means of this method it was shown that the skin possesses a specific reacting capacity for antigen more than ten times as great as the reacting capacities of muscle, brain and *in vivo* plasma; also that the extent of this capacity of the peritoneal tissues is slightly less than that of the skin.

[3] Personal communication from Dr. Florence R. Sabin.