GRADING OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES

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In attempting to define and elucidate the nature of local skin reactivity to bacterial filtrates it is of prime importance that methods be established for quantitative measurements of the reactivity. Experiments designed for this purpose and their possible application to studies on the relationship of the phenomenon under discussion to certain reactions of skin hypersensitiveness, are embodied in this paper.

Duration of Local Skin Reactivity to Bacterial Filtrates in Normal Rabbits

In early experiments on the phenomenon of local skin reactivity to bacterial filtrates it was reported that the state of reactivity disappears after 32 hours (1). Filtrates of 6 day old cultures of *B. typhosus* in tryptic digest broth were employed. Later in the work, "agar washings" filtrates were introduced, inasmuch as they were shown to contain toxic substances of considerably higher potency (2). The purpose of the work described in this part of the present paper was to study the duration of the state of reactivity induced by various preparations.

The skin of normal rabbits was prepared by a single intradermal injection of 0.25 cc. of *B. typhosus* or meningococcus culture filtrate, and after various intervals of time bacterial filtrates of ascertained reacting potency were injected intravenously. The results are summarized in Table I.

As is seen from Table I, the state of skin reactivity induced by *B. typhosus* tryptic digest broth culture filtrates disappears in 48 hours. Skin sites prepared with *B. typhosus* "agar washings" filtrates retain the reactivity for 72 hours but lose it after 96 and 120 hours. Men-

ingococcus "agar washings" filtrates induce the state for as long a period as 96 hours and occasionally for 120 hours. Skin sites prepared with *B. typhosus* "agar washings" filtrates, previously heated at

TABLE I

Duration of Local Skin Reactivity to Bacterial Filtrates

Skin-preparatory injections *	Material used for intravenous injection	Dose of intravenous injection	Interval of time between skin-preparatory and intravenous injection	Results	Reacting units per cc. of filtrate
			hrs.		
T.1832 B.Ty T_L †	T.1832 B.Ty T_L	25 reacting units	24	3/0‡	550
" "	" "	25 " "	72	1/2	550
T.1832 B.Ty T_L heated 60°—1 hr.	" "	25 " "	48	3/8	—
T.1832 B.Ty T_L heated 100°—20 min.	0.25 cc. T.1832 B.Ty T_L heated 100°—20 min.	25 " "	24	2/3	250
" "	" "	25 " "	48	0/3	250
T.1815 B.Ty T_L	T.1815 B.Ty T_L	25 " "	48	3/0	625
" "	" "	25 " "	72	1/2	625
T.1834 B.Ty T_L tryptic digest broth culture filtrate (6 days of incubation)	T.1834 B.Ty T_L tryptic digest broth culture filtrate (6 days incubation)	1 cc. undiluted	24	3/0	200
" "	" "	1 " "	48	0/3	200
" "	T.1832 B.Ty T_L	25 reacting units	24	3/0	—
" "	" "	25 " "	48	1/2	—
" "	" "	25 " "	72	0/3	—
T.1826 Mg.20745§	T.1826 Mg.20745	25 " "	24	3/0	950
" "	" "	25 " "	48	2/1	950
" "	" "	25 " "	72	2/1	950
" "	" "	25 " "	96	4/6	950
" "	" "	25 " "	120	1/2	950

* 0.25 cc. was injected intradermally.

† Abbreviation B.Ty T_L designates "agar washings" filtrates of *B. typhosus*, Strain T_L cultures.

‡ The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both indicates the total number of rabbits used in each group.

§ Abbreviation T.1826 Mg. designates "agar washings" filtrates of meningococcus cultures. The strain employed (No. 20745) was isolated from a case of cerebral spinal meningitis in this hospital.

100°C. for 20 minutes do not react for periods longer than 24 hours. Thus, the duration of local skin reactivity depends upon the preparation employed, *i.e.*, microorganism, method of preparation, etc.

As is also seen from Table I, there was determined the number of reacting units in the various preparations employed (3). Comparison of the titers with the above described results demonstrates clearly a strict parallelism between the reacting potency and the duration of reactivity induced. Preparations of low reacting potency (*B. typhosus*, tryptic digest broth culture filtrates and heated "agar washings" filtrates, containing 200 and 250 units, respectively) induce a state of reactivity of 24 hours duration. In contrast to this, *B. typhosus* "agar washings" filtrates containing 550 and 625 units elicit the reactivity for a period of 72 hours. When the intradermal injection of a *B. typhosus* preparation containing 200 units (T. 1834) is combined with the intravenous injection of "agar washings" filtrate, the reactivity lasts for 48 hours. Meningococcus "agar washings" filtrates containing 950 reacting units produce the most protracted state of reactivity (*i.e.*, 96 hours, occasionally 120 hours).

Duration of Local Skin Reactivity to Antigen-Antibody Complexes

It was previously reported (4) that the skin sites prepared by bacterial filtrates also undergo severe hemorrhagic necrosis when acted upon by toxic principles resulting from intravascular interaction of non-toxic antigens (*i.e.*, horse serum, egg albumin, etc.) with homologous antibodies. The interaction can be obtained in one of the following ways: by separate intravenous injection of antigen and the antibody; by intravenous injection of antigen into rabbits possessing actively acquired homologous antibodies; by injection of antigen into a site prepared by a bacterial filtrate with simultaneous intravenous injection of the antibody; and by injection of the antigen into the prepared skin area in rabbits possessing actively acquired antibodies. In the latter case, there apparently occurs intravascular formation of the toxic principles at the site of the locally injected antigen with the circulating actively acquired antibodies.

It was of interest to study the duration of local skin reactivity induced by "agar washings" bacterial filtrates to toxic principles formed *in vivo* through antigen-antibody interaction. Experiments were carried out as follows:

Rabbits were sensitized by single or repeated intravenous injections of horse serum. After various intervals of time indicated in Table II there were made single skin-preparatory injections of bacterial filtrates. The test injections of horse serum were given either locally into the prepared skin site or intravenously. The intervals of time between the skin-preparatory and test injections varied from 24 to 144 hours. When repeated skin-preparatory injections in the same rabbits were necessary, various bacterial filtrates were employed in order to avoid the acquirement of active immunity to the phenomenon under consideration (5). The results of the experiments are summarized in Table II.

As is seen from Table II, the reactivity induced by bacterial filtrates in horse serum-sensitized rabbits was not longer than in normal rabbits. The state elicited by meningococcus "agar washings" filtrates lasted for longer periods of time than with similar preparations of *B. typhosus*. It even appeared that the duration was decreased somewhat by previous sensitization with horse serum, since some groups prepared with meningococcus "agar washings" filtrates did not react 96 hours later. Inasmuch as the purpose of the work was to establish whether the duration of reactivity was increased by horse serum sensitization, more detailed investigation of the suggestive shortening was not made and, therefore, the question is left open.

It was also clearly seen that repeated sensitization with horse serum (1 to 3 weekly intravenous injections) did not prolong the duration of reactivity elicited by bacterial filtrates.

It is of interest to report the following experiment not recorded in Table II.

Three rabbits received each one intravenous injection of 1 cc. per kilo of body weight of normal horse serum. 48 hours later, 0.25 cc. of *B. typhosus* "agar washings" filtrates was given intradermally. 120 hours later the skin site injected with the *B. typhosus* filtrate and one unprepared skin site were each injected with 0.5 cc. of undiluted horse serum. The latter procedure was repeated four times. Following the first, second and third intradermal injections of normal horse serum into the sites prepared with bacterial filtrates marked hemorrhagic and necrotic lesions were obtained. No reactions were obtained from the injection of horse serum into unprepared sites. Following the fourth injection of horse serum, hemorrhagic necrosis appeared in all the horse serum-injected sites, which obviously were reactions of the Arthus phenomenon.

It is plain that the reactions obtained in the sites prepared by bacterial filtrates are elicited in rabbits before they become sensitive to

TABLE II

Duration of Local Skin Reactivity to Reacting Factors Formed in Vivo

Intravenous sensitizing injections			Skin-preparatory injections*			Interval of time between 1st sensitizing and various skin-preparatory injections			Test injections 24 hrs. after intradermal injections of bacterial filtrates			Interval of time between skin-preparatory and following test injections			Results of test injection		
1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
						days	*days*	*days*				*hrs.*	*hrs.*	*hrs.*			
1 cc. horse serum	1 cc. horse serum	—	T.1826 Mg.†	T.1826 Mg.	T.1832 B.Ty T_L‡	7	14	18	i. d. 0.5 cc. horse serum	i. d. 0.5 cc. horse serum	i. d. 0.5 cc. horse serum	48	72	96	4/0§	3/0	0/3
" "	—	—	T.1832 B.Ty T_L	T.1836 Mg.	—	10	20	—	" "	" "	" "	72	96	120	2/1	2/0	0/2
" "	1 cc. horse serum	—	" "	T.1826 Mg.	—	7	14	—	i. v. 1 cc. horse serum	i. v. 1 cc. horse serum	—	48	72	—	3/0	0/3	
" "	" "	—	" "	" "	—	7	14	—	" "	" "	—	96	96	—	0/3	0/3	
" "	" "	1 cc. horse serum	" "	" "	T.1836 Mg.	7	14	21	" "	" "	i. v. 1 cc. horse serum	120	120	120	0/3	0/3	0/3

i. d. = intradermally. i. v. = intravenously.

* 0.25 cc. was injected intradermally.

† Abbreviation Mg. designates "agar washings" filtrates of meningococcus cultures.

‡ Abbreviation B.Ty T_L designates "agar washings" filtrates of *B. typhosus*, Strain T_L cultures.

§ The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both indicates the total number of rabbits used in each group.

the Arthus phenomenon. The preparation with the bacterial filtrates by no means accelerates the process of sensitization to this phenomenon. Once the Arthus sensitization is obtained, the previous preparation by bacterial filtrates seemingly does not enhance the severity of the reactions.

Quantitative Measurements of Local Skin Reactivity of Normal and Horse Serum-Sensitized Rabbits

In previous experiments (2) the titration of the skin-preparatory potency of the filtrates was made as follows:

> From four to six skin sites of rabbits were injected simultaneously with various dilutions of the filtrate tested. 24 hours later the rabbits received a single intradermal injection of a given amount of the filtrate. The experiment was repeated several times and the average minimal amount of the filtrate necessary for skin preparation was computed. In view of the previously recorded quantitative reciprocal relation existing between the skin-preparatory and reacting doses, it was necessary to inject large amounts of the filtrate intravenously in order to obtain reactions in several prepared skin sites. The injection of large amounts was troublesome because of the high mortality induced.
>
> In the experiments described below, only one skin site was prepared. Each group of the rabbits tested received a different dilution of the filtrate. The intravenous dose was kept constant.

As is seen from Table III, the titrations of skin-preparatory factors of *B. typhosus* "agar washings" filtrates were tested in normal and horse serum-sensitized rabbits. The sensitization was accomplished by the intravenous injection of horse serum in a dose of 1 cc. per kilo of body weight. The injections were repeated until severe reactions appeared at skin sites prepared by bacterial filtrates 4 to 5 hours after the intravenous injection of the horse serum. Most of the rabbits acquired this sensitization 1 week after the first intravenous injection of horse serum. In some rabbits, two horse serum injections were required.

As is seen from the experiments summarized in Table III, dilutions of "agar washings" filtrates as high as 1:200 were able to elicit the state of reactivity in normal rabbits, provided 5 to 25 reacting units were injected intravenously. In horse serum-sensitive rabbits strong reactions were obtained by skin-preparatory injections of dilutions

as high as 1:150 and failed with dilutions 1:200, provided 5 to 25 reacting units were injected intravenously.

Here again, horse serum sensitization did not enhance the skin-preparatory potency of the bacterial filtrates.

DISCUSSION

It seems that before any attempts can be made to study the nature of local skin reactivity to bacterial filtrates methods should be set forth for its quantitative measurements. Experiments described in

TABLE III

Titration of Skin-Preparatory Potency of Filtrates in Normal and Horse Serum-Sensitized Rabbits

Sensitization of rabbits prior to titrations	Intradermal injections		Intravenous injections		Results
	Material	Dilution	Material	No. of reacting units	
—	B.Ty T_L* 1916	1:200	B.Ty T_L 1916	5	2/1
—	" "	1:150	" "	25	2/1
—	" "	1:200	" "	5	2/1
—	B.Ty T_L 1938	1:200	B.Ty T_L 1938	25	2/1
—	" "	1:300	" "	5	0/3
Horse serum sensitization	" "	1:60	" "	5	3/0
" "	" "	1:120	" "	5	2/1
" "	" "	1:200	" "	5	1±/2
" "	" "	1:300	" "	25	0/3

* Abbreviation B.Ty T_L designates "agar washings" filtrates of *B. typhosus*, Strain T_L cultures.

this paper demonstrate that the necessary estimations can be accomplished in a twofold manner: It is possible to determine the intensity of reactivity by studies on its duration. The duration depends upon the potency of preparations employed. It disappears within 48 hours with *B. typhosus* tryptic digest broth culture filtrates but persists for 72 hours with *B. typhosus* "agar washings" filtrates. Meningococcus "agar washings" filtrates yield a more protracted state of reactivity (up to 120 hours). Filtrates heated in the Arnold sterilizer for 20 minutes produce a state of reactivity of not longer than 24 hours duration. The various preparations were also titrated for the reacting

potency. It became obvious from these titrations that the duration of reactivity elicited by the various preparations is in direct relationship to their reacting potency.

It was previously reported that heating of the filtrates in the Arnold sterilizer at 100°C. does not destroy the phenomenon-producing power of the filtrates. It appears, however, from these experiments that the potency of the filtrates is at least partially decreased by exposure to heat, inasmuch as the duration of the reactivity elicited by filtrates heated at 100°C. for 20 minutes is considerably shorter than with unheated filtrates.

The reactivity can be also measured accurately by quantitative titrations of skin-preparatory factors against the constant intravenous dose of reacting factors. If a single skin site is prepared and 5 to 25 reacting units are injected intravenously, dilutions as high as 1:200 are able to prepare for severe hemorrhagic necrosis in a high percentage of rabbits. It is interesting to point out here that skin sites prepared with these dilutions undergo no gross inflammatory reactions prior to the intravenous injections. In most instances, it is impossible to detect the site of the preparatory injection with the naked eye. This appearance of the prepared skin site preceding the intravenous injection is in sharp contrast to the dramatic, hemorrhagic and necrotic lesion elicited a few hours after the intravenous injection. It is obvious, therefore, that the explanations of the mechanism of the phenomenon, offered by Menkin and Freund, as summations of either inflammatory or hemorrhagic effects of the two injections are untenable (6, 7).

Advantage was taken of the above experiments in order to add further evidence for differentiation of the phenomenon of local skin reactivity to bacterial filtrates from the Arthus phenomenon. As pointed out previously, there are clear-cut points of differentiation which are briefly as follows: short duration of local skin reactivity as proven again by the additional experiments presented in this paper; lack of passive transfer; short incubation period necessary for the elicitation of the reactivity, the necessity to give the test injection *via* the blood stream, specific neutralizations of the preparatory and reacting factors by immune sera; the inability to induce the

local reactivity by animal proteins; the non-specificity of the phenomenon, etc.

Recently the following observations were made. If rabbits are prepared by an injection of bacterial filtrates and 24 hours later a mixture of some animal protein with homologous antiserum is injected intravenously into the rabbits, there appears a severe hemorrhagic necrosis at the prepared skin site 4 hours after the intravenous injection of the antigen-antibody complexes. The interpretation of this experiment was that tissues exposed to the effect of certain soluble bacterial products become highly susceptible to humoral toxic principles resulting from antigen-antibody interaction. The humoral nature of these toxic principles was also clearly shown by experiments in passively sensitized rabbits. In these experiments the antigen was injected immediately or ½ hour after the injection of the antibody.

In further work it was shown that the same reactions at the sites prepared by bacterial filtrates could be obtained by the intravenous or intradermal injection of the homologous antigen into rabbits previously sensitized. In these experiments one also dealt with active acquired cellular anaphylactic sensitization. It was important, therefore, to determine whether the tissue sensitization to animal proteins could influence the state of reactivity elicited by bacterial filtrates; and also whether the reactions of the Arthus phenomenon were enhanced by bacterial filtrates.

The data presented show that the state of protein hypersensitiveness does not influence to any appreciable degree the phenomenon of local skin reactivity to bacterial filtrates. In the experiments, either bacterial filtrates or horse serum were injected into normal and horse serum-sensitized rabbits prepared by intradermal injections of bacterial filtrates. The skin-preparatory potency of bacterial filtrates and the duration of the ensuing reactivity was not increased in horse serum-sensitized rabbits. Furthermore, the sensitization to horse serum was repeated several times and the duration of reactivity was studied after each sensitizing injection. In this manner, it was shown that repeated anaphylactic sensitization did not increase the reactivity induced by bacterial filtrates. If sensitization was continued long enough and the injection of horse serum alone was sufficient for elicita-

tion of hemorrhagic necrosis, the additional injection of bacterial filtrate did not enhance the reaction.

It is obvious, therefore, that the observations deal with a certain reactivity induced by bacterial filtrates to humoral toxic principles formed intravascularly through the interaction of non-bacterial antigen-antibody complexes, and that it should be clearly differentiated from the Arthus phenomenon which may take place in the same animals after a considerably longer period of time of sensitization, and upon which the bacterial filtrates seemingly have no influence. The interest of these observations seems to lie in the fact that tissues may be so influenced by exposure to soluble antigenic bacterial substances[1] as to make them highly susceptible to intravascular interaction of antigen-antibody complexes. For many years it has been assumed that antigen-antibody complexes bring about formation of toxic principles; *i.e.*, anaphylatoxins (8, 9). It seems that the observations here reported make possible a clear-cut demonstration of the highly toxic principles resulting from these combinations and demonstrate to some extent the mechanism of their effect. Their possible rôle in immunopathology warrants serious consideration.

CONCLUSIONS AND SUMMARY

In this paper there are described methods for grading the local skin reactivity induced by bacterial filtrates. The grading is accomplished by titration of the skin-preparatory factors; and by studies on the duration of ensuing reactivity.

It was found that the duration of reactivity depends upon the mode of preparation of filtrates and the microorganisms employed. Thus, it lasts for 96 hours with meningococcus "agar washings" filtrates; for 72 hours with *B. typhosus* "agar washings" filtrates; it disappears within 48 hours with *B. typhosus* tryptic digest broth culture filtrate

[1] Thus far, it has been found that only bacterial filtrates are capable of inducing the state of reactivity of the phenomenon under discussion. Freund (7) recently injected guinea pigs with silver nitrate intradermally, and later with *B. typhosus* toxin intracardially. The tendency of silver nitrate alone to produce hemorrhages and severe necrosis in guinea pigs made the interpretation of his experiments very difficult. Silver nitrate has no skin-preparatory effect in rabbits.

and *B. typhosus* "agar washings" filtrates previously heated in the Arnold sterilizer for 20 minutes.

Comparative titrations of the preparations employed demonstrate that the duration of reactivity is in direct relationship to the reacting potency.

It is also shown that the skin-preparatory potency of the filtrates and the duration of the ensuing local reactivity are not modified by cellular anaphylactic sensitization (Arthus phenomenon) to animal proteins.

The exposure of tissues to the effect of certain soluble bacterial factors induces a high susceptibility to humoral toxic principles resulting from intravascular antigen-antibody interaction.

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