MICROBIOLOGY AND IMMUNITY

THE RELATION OF THE SITE OF ACTION OF THE ANTIGEN IN THE BODY AND THE SITE OF ANTIBODY FORMATION

(In Experimental Immunization with Tetanus Toxoid)

COMMUNICATION III. A COMPARISON OF THE VALUE OF INTRAMUSCULAR AND INTRAVENOUS INJECTION OF TOXOID AND THE IMPORTANCE IN IMMUNIZATION OF ANTIGEN CIRCULATING IN THE BLOOD

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It was shown in a previous paper [5] that the intramuscular injection of toxoid has no advantage over intravenous injection from the point of view of the concentration and duration of the presence of antigen in the blood, although it is considerably more effective in immunization. From these facts there arose the hypothesis that the basic sites of immunizing activity after intramuscular injection of toxoid are the organs and tissues which take up the antigen before it enters the blood stream, i. e., the regional lymphatic glands or the muscles. In order to provide grounds for this hypothesis it was necessary to compare the effectiveness of the intramuscular method of immunization with the effectiveness of the intravenous injection of antigen according to a scheme would imitate the gradual absorption of antigen from the muscle and also prolong its circulation to a known degree. In working out this scheme we made use of previously obtained findings [5], according to which the duration of absorption of toxoid from the muscle does not exceed 3-5 days, and moreover the greater part of the antigen is taken up during the first 24 hrs.

EXPERIMENTAL METHOD AND RESULTS

For the experiments we selected 10 rabbits of the chinchilla family, mainly males; their average weight was 2.3 kg. During the course of 4 days they were injected intravenously 12 times in the auricular vein with tetanus toxoid (series No. 336-4, obtained from the N. F. Gamaleia Institute of Epidemiology and Microbiology of the Academy of Medical Sciences, USSR), diluted with 10 times its volume of physiological saline. The total dose of toxoid injected into each rabbit was 0.5 ml in a volume of 5 ml, and the individual dose varied from 0.02 to 0.05 ml in a corresponding volume. The intervals between injections gradually increased from 2 to 24 hrs.

Another group of animals (7 rabbits of the same sex and weight) were given a single injection of 0.5 ml of undiluted toxoid into the rectus femoris muscle. Part of the animals (3 rabbits) received in addition intravenous injections of physiological saline according to the same scheme followed in immunizing the animals of the first group.

Investigation of the toxoid content of the blood serum of the experimental animals by a modification of Becher's [4] method showed that the antigen circulated for a longer time in the blood of the first group of rabbits than after intramuscular immunization. Two months later all the animals were revaccinated intravenously with a single injection of 0.5 ml of toxoid.

After the primary immunization, and also after revaccination systematic estimations were made of the antitoxin content of the blood sera of the animals by the usual titration in mice. The results obtained are shown in Fig. 1.

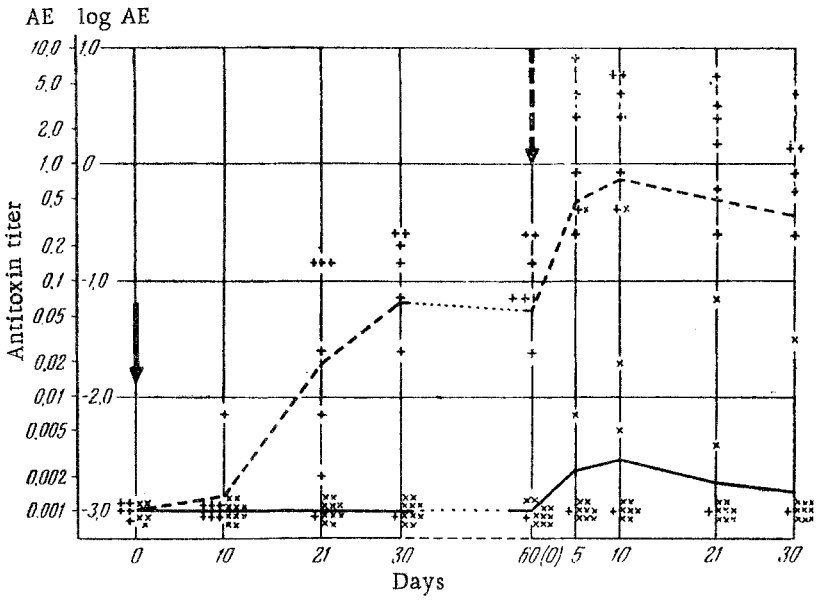

Fig. 1. Comparative effectiveness of fractional intravenous and single intramuscular immunization. Conventional signs: ↓) primary immunization; x) individual antitoxin titers after fractional intravenous injection of antigen (primary immunization); ——) their geometric mean; +) individual antitoxin titers after intramuscular injection of antigen (primary immunization); ---) their geometric mean; ↓) revaccination (intravenously). Here, and in Fig. 2, antitoxin titers below 0.002 antitoxin units (threshold of sensitivity of the method) are conventionally indicated as being equal to 0.001 units. AE - Antitoxin units.

As can be seen in Fig. 1, fractional intravenous immunization did not cause antibody formation in one of the 10 rabbits, and the majority of the animals (7 out of 10) remained indifferent also to the revaccination injection of antigen.* In the majority of rabbits (6 out of 7) immunized intramuscularly, however, antibody formation was observed; subsequent intravenous injection of antigen brought about in these animals the characteristic rapid rise in the antitoxin titer associated with revaccination.

Thus injection of toxoid directly into the blood stream caused the formation of antibodies only in the presence of previous immunizational reorganization of the body as a result of intramuscular immunization, but could not as a rule bring about this reorganization itself despite the prolonged circulation of the antigen in the blood. A similar enhancement of the intravenous immunization effectiveness as a result of previous injection of antigen into the tissues is also known in the case of diphtheria toxoid [8].

However, the possibility was not excluded that during intramuscular injection an altered antigen is absorbed into the blood, more active so far as immunization is concerned. Although in investigation of the revaccinating

* In assessing the possible causes of the rise in antitoxin titers observed in certain rabbits after revaccination, one has to take into consideration the unavoidable leakage of small quantities of antigen into the tissues in the process of repeated intravenous injections. As shown later, injection into the tissues of a subthreshold dose of antigen in conjunction with intravenous injection of toxoid causes a satisfactory immunizing effect. Additional data in support of such a view will appear in one of the later reports.

activity of sera from blood taken after intramuscular or intravenous immunization were not essentially different [5], this possibility mentioned must be checked once more by another method. For this purpose we examined the immunizing effect of intravenous injection of toxoid previously incubated in muscle in vivo.

We injected 11 donor rabbits of both sexes with 1.5 ml of toxoid into the right and left rectus femoris muscles. These muscles were excised after an interval of time from a few minutes to 24 hours, and then were ground up for 5 min in 3 times their volume of physiological saline in a Waring type blender (made in the experimental factory of the Academy of Medical Sciences, USSR). The ground-up mass was centrifuged for 7-8 min at 2500 rev/min; the sediment was discarded. All the operations were performed as quickly as possible, with sterile precautions, and in the cold; penicillin was added to the prepared extracts in a dose of 3000 IU per 1 ml. The extracts were injected intravenously into 5 recepient rabbits according to the 12 injection scheme described above, and the schedule of injections of the extracts corresponded in duration to the incubation of the antigen in the muscle of the donor. A control group of recipients (3 rabbits) was immunized intramuscularly with 0.5 ml of

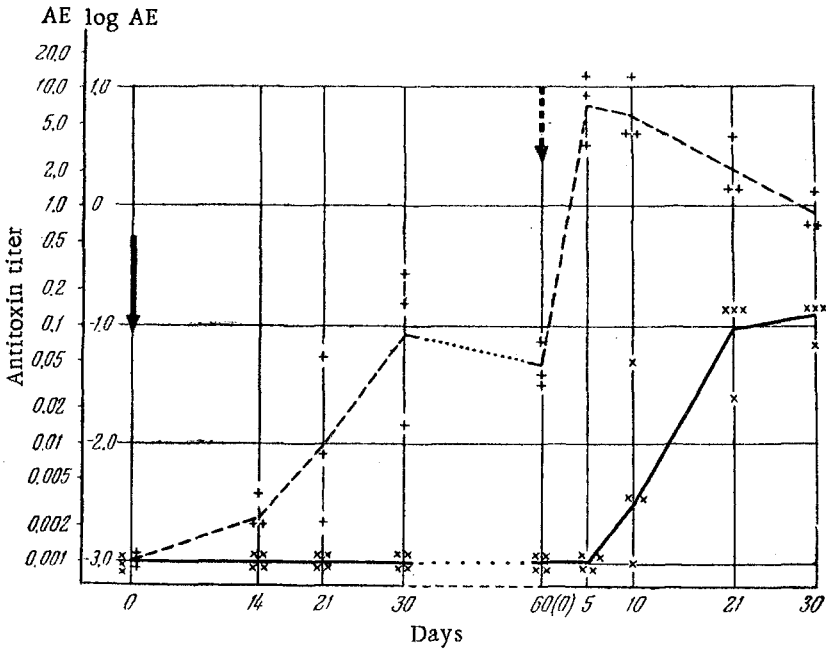

Fig. 2. Effectiveness of intravenous immunization with toxoid incubated in muscle. Conventional signs: ↓) primary immunization; x) individual antitoxin titers of the experimental group of rabbits; ——) their geometrical mean; +) individual antitoxin titers of control animals immunized intramuscularly with native antigen; ---) their geometrical mean; ↓) revaccination (intramuscularly). AE- Antitoxin units.

native toxoid; in order to equalize the experimental conditions this group of animals received intravenously a muscle extract from an unimmunized donor. The total dose of extract amounted to 45 ml for each rabbit, the individual doses from 2 to 5 ml. Injection of the extract as a rule caused no apparent reactions (rarely slight shortness of breath). The only exception was one rabbit of the experimental group which died 10-15 min after the 10th injection. At necropsy extensive recent hemorrhages were found beneath the endocardium of the ventricles (particularly the right) and widespread areas of hepatization in the lungs.

Judging by the antitoxin-combining activity of the extracts, each recipient in the experimental group received a dose of antigen equivalent to 0.6± 0.3 ml of native toxoid. In the blood sera of the recipients of the first group the antigen content either was approximately equal to (24 hrs after immunization) or greater than (3 days afterwards) the corresponding values in the control group.

After 60 days all the recipient rabbits were revaccinated intramuscularly with 0.5 ml of toxoid. Their blood antitoxin content was systematically investigated (after both primary immunization and revaccination). The results obtained are shown in Fig. 2.

As can be seen in Fig. 2, rabbits immunized intramuscularly with native toxoid showed the typical effect of primary immunization with maximum titers on the 30th day, and after the subsequent injection of antigen – a sharp revaccination rise in the titers with a maximum on the 5th-10th day. In those rabbits, however, which were immunized with intravenous toxoid previously incubated in muscle, antitoxin production did not take place, and subsequent intramuscular injection of antigen caused only the ordinary primary immunization effect. Thus the antigen circulating in the blood stream in these experiments had no power to cause either antibody formation or to increase the reactivity of the animal to subsequent injection of antigen.

It does not follow from this, however, that toxoid circulating in the blood is in general unable to play any part in the formation of the primary immunization reaction. This is shown convincingly by the results of simultaneous intravenous and intramuscular immunization of rabbits. The first group of animals were given daily injections for four days of tetanus toxoid, diluted 5 times with physiological saline, into the marginal vein of the ear. The total dose of toxoid for each rabbit was 0.5 ml in a volume of 2.5 ml; the individual doses were gradually reduced from 0.16 to 0.06 ml. The second group of rabbits were given a single injection into the rectus femoris muscle of undiluted toxoid in a dose of 0.5 ml. The third group of animals received a single intramuscular injection of 0.15 ml of toxoid diluted to 0.5 ml. Finally, the fourth group of animals also received 0.15 ml of toxoid diluted to 0.5 ml intramuscularly and in addition 0.35 ml of toxoid intravenously (the first injection was given at the same time as the intramuscular injection) by a scheme similar to that used in the immunization of the first group of rabbits. To make the experimental conditions equal, animals immunized intramuscularly were given additional intravenous injections of physiological saline, and vice versa. After 3 weeks, as shown by investigation of the antitoxin titers (see table), the animals of the second and fourth groups showed a well-marked immunization reaction, those of the third group a very weak reaction and those of the first group no reaction at all. It follows from these findings that although by itself the toxoid circulating in the blood (in accordance with the findings of the preceding series of experiments) has no power to cause the formation of antibodies, an increase in its concentration intensifies the effect of intramuscular immunization with a subthreshold dose.

The Immunizing Effectiveness of Fractional Intravenous and Single Intramuscular Injection of Antigen, and of Their Combined Administration

Group of animals	Scheme of immunization	Number of rabbits	Immunizing effect on the 21st day (M± 2m)	
			% of animals with antitoxin in the blood (≥ 0.002 antitoxin units)	average titers in antitoxin units
First	0.5 ml of toxoid intravenously (fractional)	5	0^{+33}_{-0}	< 0.002
Second	0.5 ml of toxoid intramuscularly	12	83^{+17}_{-23}	0.120± 0.063
Third	0.15 ml of toxoid intramuscularly	14	36± 27	$0.016^{+0.022}_{-0.016}$
Fourth	0.15 ml of toxoid intramuscularly + 0.35 ml of toxoid intravenously (fractional)	14	86^{+14}_{-18}	0.111± 0.087

Thus the high effectiveness of the intramuscular method of immunization compared with the intravenous cannot be explained either by the longer circulation of antigen in the blood nor by any form of activation of the antigen in the muscle tissue. Toxoid injected into the blood stream in conditions which imitate its natural absorption was found as a rule to be incapable of causing any alteration in the immunological reactivity of the animal. The decisive factor in bringing about the primary immunization reorganization of the body must therefore be regarded as the action of the antigen on those organs and tissues penetrated by the toxoid before it enters the general circulation. In intramuscular immunization these organs may be the muscles or regional lymphatic glands. According to some reports [14], tetanus toxin, like other proteins with a molecular weight over 20,000, enters the circulation mainly, or even entirely, by the lymphatic route. Evidently the same must occur with absorption of tetanus toxoid, whose molecular weight is not less than 68,000 [12]. The low power of penetration of the blood capillaries by toxoid is shown by its prolonged deposition in the muscle and its circulation in the blood [5].

Analyzing the possible causes of the narrow localization of the initial phases of the immunization activity, it must be pointed out that the higher effectiveness of immunization by injection directly into the tissues compared with the intravenous method is also characteristic of other molecularly dispersed antigens [1, 3, 6, 7, 8, 11, 15], irrespective of their specific properties. However, in the case of corpuscular antigens (erythrocytes, killed cultures of bacteria, and so on) a number of writers [1, 2, 10, 16] found the converse to be true. There are also reports [7] of the increased effectiveness of intravenous injection of antigen if it is adsorbed on alumogel. It seems likely that the low immunizing activity of a molecularly dispersed antigen circulating in the blood stream is due to a considerable extent to the low concentration of antigen compared with that arising in the tissues and lymphatic system after the intramuscular method of injection. The correctness of this attitude to the question is confirmed by the findings of Glenny and his co-workers [9] who observed a considerable reduction in the immunizing effect by dilution of diphtheria antigen injected intramuscularly with physiological saline. On the basis of the general physiological ideas of a threshold of excitability of a biological structure, it might be thought that the reorganization of the body in primary immunization cannot be brought about by the taking up of solitary molecules of antigen even by a large number of cells; it demands, on the contrary, the action of a sufficiently massive dose of antigen even though the number of appropriate functional units (cells forming antibody, or nerve endings) is restricted.* It is clear that in the case of immunization with corpuscular vaccines this condition is satisfied also during their intravenous injection. In the course of the immunizational reorganization of the body the sensitivity to the antigen rises sharply; thus in our experiments [5] the injection of toxoid diluted even 1000 times had a significant revaccinal effect. This makes clear the effectiveness of intravenous revaccination mentioned above. These ideas which we have discussed may be of value in the future study of the mode of action of the so-called adjuvants.

SUMMARY

The content of toxoid was investigated in the blood serum in certain methods of intravenous and intramuscular immunization. The period of circulation of the antigen in the blood as well as its possible qualitative changes were examined. The data obtained permit the belief that toxoid circulating in the blood is of secondary significance in the primary immunizational reactions of the organism. The effect of antigen on the regional lymph nodes or on the tissues at the site of injection should be considered as a decisive factor. The question of dependence of the place of action of the antigen on its physicochemical structure is discussed.

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* As shown by the investigations of Leduck et al. [13], there is a marked disproportion between the large number of cells taking up antigen and the small number of cells producing antibody.

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THE USE OF THE REACTION OF INCREASE OF PHAGE TITER IN THE DETECTION OF FLEXNER DYSENTERY BACTERIA IN INFECTED RABBITS

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The spread of dysentery bacteria throughout the human body is of considerable interest from the point of view of solving problems of the pathogenesis and also of specific prophylaxis of dysentery. The views expressed in the literature on the spread of dysentery microorganisms in the body are based on bacteriological detection of the agent in the blood or in organs of persons dying from dysentery [3, 4], and on bacteriological examinations of the blood and bile during life (Denner, Kulesha, Besredka et al.).

In recent years series of experiments have been performed to study the pathogenesis by means of radioactive isotopes. V. I. Ivanov and his co-workers [2] describe their findings of the distribution in animals of antigens of typhoid bacteria containing radioactive phosphorus P^{32}. V. L. Troitskii and his co-workers [8], using Flexner-Sonne dysentery vaccine labeled with radiophosphorus, demonstrated rapid penetration of microorganisms into the blood stream after infection of rabbits per os. The results so far obtained suggest that other methods of indication of dysentery microorganisms in the body are necessary in order to elucidate the problems of pathogenesis and immunity in dysentery.

In the present paper we describe an experimental study of the possibility of using the reaction of increase of phage titer (the method of V. D. Timakov and D. M. Gol'dfarb) to detect dysentery microorganisms in infected rabbits. This reaction was widely tested by these authors for diagnostic purposes in detecting dysentery bacteria in the stools of patients and carriers, and also in various environmental objects (in water, and washings from the hands and various utensils).

EXPERIMENTAL METHOD

The principle of the reaction is based on the increase in the titer of highly sensitive indicator phage during its interaction with dysentery bacteria contained in the material for examination. It enables these microorganisms to be found in a short time (10-20 hrs) in very low concentrations. The experiment was performed on 53 rabbits, divided into 4 groups. The animals were infected with a mixture of dysentery strains of Flexner a, b and c types, whose virulence, expressed LD_{100}, was equal to 500 million bacterial bodies per 1 ml. For infection we used washings in physiological saline of 24-hr cultures with an optical standard of 1 and $4 \cdot 10^9$ bacterial bodies per 1 ml.