RELATIONSHIP BETWEEN THE SITE OF ANTIGEN ACTION IN THE BODY AND THE SITE OF ANTIBODY PRODUCTION (AS EXEMPLIFIED BY IMMUNIZATION WITH TETANUS ANATOXIN)

COMMUNICATION II. DYNAMICS OF TETANUS ANATOXIN RESORPTION IN THE BODY USING INTRAMUSCULAR AND INTRAVENOUS IMMUNIZATION

L.N. Fontalin

Laboratory of Infectious Pathology (Director – Corresponding Member Acad. Med. Sci. USSR A.Ia. Alymov), Department of General Pathology (Director – Academician A.D. Speranskii), Institute of Normal and Pathologic Physiology (Director – Active Member Acad. Med. Sci. USSR V.N. Chernigovskii), Acad. Med. Sci. USSR, Moscow

(Received September 25, 1957. Presented by Active Member Acad. Med. Sci. USSR N.N. Zhukov-Verezhnikov)

In recent years there has been an extreme growth of interest, both in the Soviet Union and abroad, as to the questions of antigen resorption in the body. This is not surprising: the site of introduction of the antigen, the routes and rate of its further spread, duration of its presence in the general circulation and in various organs and tissues (being the origin of the developing immunizing process) determine the latter's character and intensity to a considerable degree. Therefore, investigation of antigen resorption in the body becomes one of the most important forms of analysis of processes of immunity formation.

Literature data on resorption of tetanus anatoxin in the body are very scant. Thus, Ramon and Felchetti [12], using indirect methods, showed that as early as the first nine hours after subcutaneous injection a considerable amount of anatoxin is resorbed into the bloodstream. After injection of alum-precipitated anatoxin its presence at the site of injection could be traced for a period of 20 days [1]. It is known [13] that the dynamics of resorption of even closely related (physicochemically and immunologically) antigens may be quite different. Glenny's [11] and Christensen's [9] data must be mentioned, with these reservations; according to these authors intramuscularly injected foreign serum protein enters the blood chiefly during the first three days after the injection.

The present work is concerned with investigation of the time relationships between the presence and content of tetanus anatoxin at the site of injection and in the blood serum of animals following intramuscular and intravenous immunization. In view of the marked difference in effectiveness of these methods of immunization [4, 6], parallel investigation of the antigen resorption processes could prove to be valuable with respect to selection of the site of antigen injection, the distribution of the substrate reacting to the antigen and the temporal norms of the immunizing action.

EXPERIMENTAL METHOD

All the experiments were performed on rabbits (chinchilla variety) of both sexes (predominantly male), weighing from 1.6 to 2.9 kg; the experiments were carried out at different times of the year. Immunization was achieved with tetanus anatoxin series No. 336-4 (received from the N.F. Gamalei Institute of Epidemiology and Microbiology), containing 75 antitoxin fixation units per 1 ml. The anatoxin was given in the dose of 0.5 ml into the rectus femoris muscle or into the pinna vein.

In the first series of experiments the antigen content at the site of injection (muscle) was investigated one to three days after immunization. This was achieved by extirpation of the muscle at appropriate intervals under sterile conditions; the muscle was then homogenized in a glass homogenizer* at 300 rpm, in a volume of physiologic solution three times that of the weight of the muscle, until disappearance of visually perceptible tissue particles. The whole operation was performed at 0°. Absence of anatoxin destruction in the process of homogenizing was shown in special experiments. The concentration of the anatoxin in the homogenates was determined by the Becher method modified as by us [8], administering the homogenates to mice in admixture with equilibrated amounts of tetanus anatoxin (1/50 L) and antitoxin (0.004 AU/ml). Control groups of mice received under similar conditions muscle homogenates from unimmunized rabbits (pure control) and with admixture of anatoxin in various dilutions (control with anatoxin). Comparison of the severity of intoxication of the experimental and control groups of mice permitted conclusions concerning the presence and concentration of anatoxin in the homogenates under investigation and hence concerning the antigen content of the muscles.

EXPERIMENTAL RESULTS

As can be seen from Table 1, 24 hours after immunization the muscle still contains 25 ± 15% of the antigen introduced into it. Anatoxin was found in the muscles of all the eight rabbits examined, the individual determinations giving results which agreed with the value cited above. It may thus be considered as established that a temporary anatoxin depot is formed in the muscle following introduction into it of the usual immunizing dose of anatoxin; this depot is almost completely depleted between the first and third day after immunization, although traces of anatoxin are evidently still present after this interval. This latter fact required further investigation in the light of the consistency of temporal summation of antigen action on the body and its possible significance in the mechanism of high effectiveness of intramuscular immunization as compared with intravenous.

In order to detect traces of antitoxin in muscles during the late stages of resorption (three to five days) we used a revaccinating test based on the high sensitivity of immunized animals to repeated administration of the antigen.** Muscle homogenates from immunized rabbits were prepared as described above and were kept in the cold (at 0°) for one to four days after which they were strained through several layers of sterile gauze; the filtrates obtained were centrifuged for three minutes at 2-3000 rpm, the supernatant liquid was collected and the deposit discarded. The muscle extracts so obtained (constituting about 1/4 of the original homogenates by volume) were introduced into the quadriceps femoris of the recipient rabbits immunized two to five months prior to the experiment with 0.5 ml anatoxin given into the same muscle. Each recipient rabbit received the whole of the muscle extract from the corresponding donor (3.5-7 ml). The use of extracts was connected with the fact shown by previous experiments in which administration of whole homogenates caused abscesses in the recipients. Special experiments showed that concentration of anatoxin in the extracts corresponded to the anatoxin concentration in the homogenates from which they were prepared. Ten days after administration of the extracts the recipients' blood serum antitoxin titer was determined and compared with the titer before extract administration. Recipients given muscle extracts from unimmunized rabbits served as controls.

Table 2 shows that extracts from muscles extirpated three days after immunization produced in the majority of recipients a characteristic revaccination effect. This can also be taken to confirm the presence of traces of antigen in the muscle three days after immunization. When extracts of muscles extirpated five days after immunization were used, however, the majority of recipients did not react perceptibly to administration of the extracts. The high sensitivity and specificity of the test used must lead to the conclusion that resorption of anatoxin from muscle is practically finished (i.e., from the point of view of its role in formation of the immunization effect) after three to five days following immunization.*** However, the possibility is not excluded that the traces of antigen found after three to five days are accounted for by admixture of blood which, as will be shown below, also contains anatoxin during this period. The true duration of antigen resorption from the muscle can therefore be somewhat shorter, but in any case must be over 24 hours.

---

*For description of apparatus and technique of homogenizing see [7], Ch. XI.

**After finishing the present series of experiments we became aware that a similar method was used by Volgin [1].

***It is known [3] that sensitivity to repeated administration of antigen after short intervals of time (5-15 days) is much lower than after two to six months. Applying this rule to our material it is possible to conclude that if the anatoxin content of muscle five days after immunization is insufficient to elicit a revaccination effect in recipients it is all the more insufficient for participation (being resorbed into the blood stream) in formation of immunity in donors.

The next problem was to discover the way in which the temporary deposition and subsequent resorption of the depot reflected on the dynamics of blood anatoxin content. This problem was particularly important in view of the widespread opinion that the effectiveness of intramuscular (and also subcutaneous) method of immunization as compared with intravenous is connected with the more prolonged circulation of antigen. In order to clarify this question we made a comparative study of antigen concentrations in the blood of rabbits immunized intramuscularly and intravenously at different intervals (one to five days) after immunization. In the first experiment of this series ten rabbits were immunized with tetanus anatoxin, five intramuscularly and five intravenously. Blood specimens were taken one to three days later and the sera obtained were given separately to mice in admixture with equilibrated doses of toxin and antitoxin. Comparison of the severity of intoxication in these mice and in control animals who had received blood sera taken prior to immunization under analogous conditions, provided evidence for the presence of anatoxin in the sera being tested. Comparison of the severity of intoxication among the various groups of mice also permitted determination of whether the blood of rabbits immunized intramuscularly contained more or less antitoxin than blood of rabbits immunized intravenously. Subsequently additional investigations were made in a similar way of blood sera from a further eight rabbits (four immunized intramuscularly and four intravenously) taken three days after immunization. Since the results proved to be identical the data obtained have been pooled (upper half of Table 3). In the third experiment of the present series a comparison was made between the antitoxin fixation activity of sera taken from four rabbits three to five days after intramuscular immunization. Since, during this experiment, the reactivity of the mice to the toxin proved to be lowered, the results obtained, together with the control ones, are presented separately in the lower part of Table 3.

As shown in Table 3, anatoxin is found in the blood serum of animals over a period of three days, regardless of whether they were immunized intramuscularly or intravenously. One day later its concentration in the blood of animals immunized intramuscularly is noticeably lower than in those immunized intravenously. After three days this difference is evened out to some extent and, although the previous sign remains, scatter of material throws doubt on its authenticity. Finally, by the fifth day concentration of antigen in the blood of rabbits immunized intramuscularly drops sharply to levels which cannot be detected by the present method. Determination of absolute concentrations of antigen in blood is not the special problem of this investigation. Nevertheless, it proved possible in some cases to establish the order of this value with the help of a scale derived from data of control experiments (not shown in table): in blood serum taken one day after intramuscular immunization — 0.03-0.15 AFU/ml* (the most probable concentration 0.05-0.08 AFU/ml); after three days — less than 0.08 AFU/ml (most probable concentration 0.03-0.05 AFU/ml); one day after intravenous immunization — above 0.08 AFU/ml (most probable concentration 0.1-0.15 AFU/ml); after three days — value of the same order as after intramuscular immunization.

Thus, at none of the intervals tested (up to the limits of the method's sensitivity) were higher concentrations of antigen found in the blood of animals immunized intramuscularly as compared with those immunized intravenously. This fact had to be checked with the help of a more sensitive method and at time intervals at which the former method proved ineffective, i.e., five days after immunization. The revaccination test was used for this purpose: the sera under investigation were introduced (1 ml) into the recipient-rabbits' quadriceps muscle; the rabbits had been immunized with tetanus anatoxin three to four months prior to this procedure. As can be seen from Table 2, only half the recipients showed the characteristic revaccination effect, regardless of the method of immunizing the donors. Consequently, the absence of substantial difference in blood anatoxin content of the animals immunized intramuscularly and intravenously can be considered as confirmed.

Comparison of results obtained on determination of anatoxin content of muscle and blood shows good correspondence. Retention of part of the antigen in the muscle during the first 24 hours determines its lower concentration in the blood as compared with intravenous immunization. Subsequently, the concentrations of antigen in the blood become evened out in the two methods of immunization, which is explained by parallel disappearance of antigen from the muscle. The fact that temporary retention of antigen in the muscle does not affect the duration of circulation of antigen in the blood can be explained by the relatively short duration of this retention and also probably by partial binding and destruction of antigen by muscle tissue and regional lymphatic elements.

It is important to note that the duration of circulation of tetanus anatoxin in the blood approaches that of other molecularly-dispersed protein antigens [6, 9, 10] and is considerably longer than circulation of corpuscular vaccine antigens [2] or so-called complete antigens [5]. Parallel with determination of blood antigen content in

*AFU — antitoxin-fixation unit.

TABLE 1

Determination of Tetanus Anatoxin Content at Site of its Injection (Muscle) One to Three Days After Immunization

Number of rabbits	Sex	Average wt. in kg	Days between extrip. and immuniz.	Average muscle wt. in g	Nature of experiment	Dilution of homogenates	No. of mice	Results of experiment (M±2m)			Anatoxin concent. in homogenates	Aver. anatoxin content in muscles % of amount administered
								% dead	survival time of animals which died (days)	general index of intoxication*		
8	5 ♂♂ 3 ♀♀	2.2	1	6.3	Administration of muscle homogenates to mice in admixture with 1/50 L_f of toxin and 0.004 AU/ml antitoxin	1/2	18	$100\frac{+0}{-10}$	3.4±0.6	7.1±0.6	>1/500	25±15
						1/4	22	$95\frac{+5}{-10}$	4.5±0.5	5.9±0.5	<1/125	
11	8 ♂♂ 3 ♀♀	2.2	3	6.4		Undiluted	28	25±17	5.2±0.6	2.8±0.6	<1/1 000	<5
Control	Muscles of four unimmunized rabbits						20	$5\frac{+10}{-5}$	—	2.1±0.5	—	—
	Ditto + anatoxin in 1/1000 dilution						20	80±18	4.6±0.5	5.3±0.7	—	—
	Ditto + anatoxin in 1/500 dilution						10	$100\frac{+0}{-18}$	3.6±0.4	6.9±0.4	—	--

*In conventional units [8].

TABLE 2

Revaccination Test for Presence of Anatoxin in Muscles and Blood Sera

Number of donor-rabbits	Sex	Average wt. in kg	Method of immunization	Days between muscle extirpation (correspondingly to blood) and immunization.	Nature of expt.	Number of recipient-rabbits	Aver. antitoxin liter (in AU/ml) in recipients		Number of recipients showing raised titer				
							prior to administration of extracts (correspondingly to sera)	after 10 days	<0.7 times	0.7—1.5 times	1.6—3.0 times	3.1—10 times	>10 times
5	3 ♂♂ 2 ♀♀	2.3	Intramuscularly	3	Administration of muscle extracts to recipients	5	0.09	0.84	—	—	1	1	3
7	6 ♂♂ 1 ♀	2.3	Intramuscularly	5	Administration of muscle extracts to recipients	7	0.12	0.29	1	3	2	—	1
7	♂♂	2.2	»	5	Administration of blood sera to recipients	7	0.29	0.60	—	1	3	1	2
8		2.1	Intravenously	5	Administration of blood sera to recipients	8	0.17	0.40	—	3	1	2	2
Control	Muscle extract from four unimmunized rabbits					5	0.12	0.09	1	3	1	—	—
Control	Rabbit sera taken before immunization					7	0.16	0.20	—	4	3	—	—
Control	Tetanus anatoxin in 1/1000 dilution					6	0.12	1.23	—	—	1	2	3

TABLE 3

Determination of Relative Content of Tetanus Anatoxin in Rabbit Serum After Intramuscular and Intravenous Immunization

Method of immunization	No. of rabbits	Aver. wt. (kg)	Days between blood specimen and immun.	Nature of expt.	No. of mice	Results of expt. (M ±2m)		
						% dead	survival of animals which later died (days)	general index of intoxications
Intramuscular	5	2.5	1	Sera to mice admixture with 1/50 L_t toxin and 0.004 AU/ml antitoxin	10	$100\frac{+0}{-18}$	3.7±0.3	6.8±0.3
Intravenous	5	2.3			10	$100\frac{+0}{-18}$	2.9±0.4	7.6±0.4
Intramuscular	9	2.2	3	Ditto	36	86±12	4.7±0.4	5.3±0.5
Intravenous	9	2.2			36	$94\frac{+6}{-8}$	4.4±0.4	5.9±0.5
Control	Blood sera before immunization			» »	16	25±22	6.0±0.7	2.8±0.6
	Ditto + anatoxin in 1/1000 dilution				14	$100\frac{+0}{-13}$	3.5±0.4	7.0±0.4
Intramuscular	4	2.5	3	» »	10	$80\frac{+20}{-27}$	5.5±0.6	4.6±0.7
			5		10	$0\frac{+18}{-0}$	—	2.0±0.2
Control	Blood sera before immunization			» »	10	$0\frac{+18}{-0}$	—	1.9±0.3
	The same + anatoxin in 1/1000 dilution				6	$100\frac{+0}{-29}$	3.8±1.3	6.7±1.3

rabbits immunized intramuscularly and intravenously we investigated selectively the antitoxin titer in the same animals on the 21st day after immunization. In agreement with previously obtained data no antibodies were found in any of the five rabbits immunized intravenously, whereas all nine rabbits immunized intramuscularly had an average blood antitoxin titer of 0.20 ± 0.05 AU/ml. No immunizing effect followed intravenous immunization despite the fact that anatoxin was circulating in the rabbits' blood and five days later could still elicit a clear revaccination effect in other rabbits when the serum was given intramuscularly (Table 2).

Analysis of material obtained allows the conclusion that, despite temporary deposition of part of the antigen when immunization is effected intramuscularly, the high effectiveness of this method of administration of antigen as compared with intravenous cannot be explained by special dynamics of circulation of antigen in the blood. It may be postulated in connection with this that in primary intramuscular immunization the action of the antigen impinges on organs and tissues reached by the antigen prior to its entry into the general circulation, in particular the regional lymphatic apparatus. Further proof of this hypothesis will be furnished in our next communication.

SUMMARY

The content of anatoxin was examined at the site of its injection (the muscle) and in the blood serum in one to five days after intramuscular and intravenous immunization. Temporary storage of a certain part of the antigen was noted in the muscle within one to three days. This antigen could be demonstrated in the blood for a period of three to five days. Its intramuscular injection during that period had no promoting effect on the increase of the antigenic concentration in the blood. Therefore, it is assumed that the higher efficacy of the intramuscular method of immunization (as compared with the intravenous) is connected with its action on the regional lymphatic apparatus and not with the more prolonged circulation of the antigen in the blood.

LITERATURE CITED

[1] I.B. Volgin, The Significance of the Principle of "Depot" and of Various Methods of Antigen Administration for Active Immunization Against Tetanus,* Thesis (Moscow, 1955).

[2] S.I. Ginzburg-Kalinina, in the book: Transactions of the Mechnikov Scientific-Research Institute of Vaccines and Sera* Vol. 8 (Moscow, 1956), pp. 160-170.

[3] P.F. Zdrodovskii, The Problem of Reactivity in Study of Infection and Immunity*(Moscow, 1950).

[4] R.I. Zel'manovich and D.F. Pletsityi, Zhur. Mikrobiol., Epidemiol. i Immunobiol. No. 1, 69-71 (1953).

[5] V.I. Ivanov, M.M. Priselkov, S.I. Cherniakhover and S.V. Lesniak, Zhur. Mikrobiol., Epidemiol. i Immunobiol. No. 11, 71-75 (1954).

[6] O.Ia. Ostryi and L.N. Fontalin, Zhur. Mikrobiol., Epidemiol. i Immunobiol. No. 1, 43-49 (1956).

[7] V. Umbreit, R.H. Burris and J.F. Strauffer, Manometric Methods in Studies of Tissue Metabolism [Russian translation] (Moscow, 1951).

[8] E.N. Fontalin, Biull. Eksptl. Biol. i Med. 44, No. 10, 100-105 (1957).**

[9] P.E. Christensen, Acta path. microbiol. Scandinav. 31, 262-274 (1952).

[10] F.J. Dixon, P.H. Maurer and M.P. Deichmiller, J. Immunol. 72, 179-186 (1954).

[11] A.T.B. Glenny, in the book: A System of Bacteriology (London, 1931), Vol. 6, pp. 106-193.

[12] G. Ramon and E. Falchetti, Compt. rend. Soc. Biol. 118, 1044-1047 (1935).

[13] R.H. Regamey, Schweiz. Ztschr. allgem. Path. u Bakt. 16, 873-881 (1953).

*In Russian.

**Original Russian pagination. See C.B. translation.