THE EFFECTS OF NITROGEN MUSTARD (METHYL BIS (β-CHLOROETHYL) AMINE HCl) ON THE IMMUNOLOGICAL RESPONSE OF THE RABBIT. I. THE EFFECTS OF NITROGEN MUSTARD ON THE PRIMARY RESPONSE TO A BACTERIAL ANTIGEN

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SINCE Gilman and Philips (1946) reviewed the effects of the nitrogen mustard group of substances on the tissues of animals, various authors (Philips *et al.*, 1947; Spurr, 1947; Bukantz *et al.*, 1949; Schwab *et al.*, 1950; Marshall and White, 1950) have described the effects of nitrogen mustard on the production of antibodies, using as antigens either soluble proteins or toxins or large or multiple injections of bacterial suspensions by the subcutaneous, intramuscular or intravenous routes. They showed that under these various experimental conditions, there is a delay in the appearance of antibodies; that titres tended to be lower when antigens were injected following doses of nitrogen mustard; that when the antigen was injected 2–3 weeks before the nitrogen mustard there was no increase in circulating antibodies following the injection of the nitrogen mustard.

The present study was undertaken to observe the effect of a standard course of nitrogen mustard on the production of antibody stimulated by one dose of a bacterial suspension (100 million *Salm. typhi* H cells) injected intravenously at varying times with respect to the nitrogen mustard course. Total and differential leucocyte counts were performed to see whether they might have any relation to the production of antibody.

MATERIALS AND METHODS

Salm. typhi suspension.—This was prepared from a laboratory strain which was rendered highly motile by the method of culture in semisolid agar in a double tube. The organisms were harvested in 0·5 per cent formol saline and stored at 4° in a concentration of 2000 million organisms/ml. using Brown's Scale. The suspension was diluted to give 100 million organisms/ml. for intravenous injection.

Nitrogen mustard solution.—Methyl bis (β-chloroethyl) amine HCl (Mustine-Boots), hereafter called nitrogen mustard, was used in the experiments. Since this compound is unstable after 30 min. when dissolved in normal saline, the solution was made up and the animals injected without delay. The solution contained 0·5 mg./ml. nitrogen mustard and the injection was made into the marginal ear vein of the rabbits using a dosage of 1 mg./kg. body weight. The rabbits used weighed 2 kg. approximately.

Schedule of dosage of nitrogen mustard.—The schedule of dosage used was modified from that employed by Schwab *et al.* (1950). A course of four doses of 1 mg. nitrogen mustard was injected at daily intervals in six groups of rabbits, each group containing 3 animals. One group of 3 animals received no nitrogen mustard.

The injection of *Salm. typhi* suspension varied from 72 hr. before the beginning of the course to 24 hr. after the end of the course.

Table I shows the schedules used.

TABLE I.—*Schedule of Dosage of Nitrogen Mustard Used in Experiment and Times of Injecting Antigen*

Animal group	Time in days nitrogen mustard course							
I	A	—	—	—	—	—	—	—
II	A	—	—	D	D	D	D	—
III	—	A	—	D	D	D	D	—
IV	—	—	A	D	D	D	D	—
V	—	—	—	D A	D	D	D	—
VI	—	—	—	D	D	A D	D	—
VII	—	—	—	D	D	D	D	A

A = 100 million *Salm. typhi* H intravenously.
D = 1 mg. methyl bis (β-chloroethyl) amine HCl/kg. body weight.

Agglutination tests.—Blood was drawn off from the marginal ear vein, the serum separated and doubling dilutions made from 1/12·5 upwards in 0·25 ml. amounts and 0·25 ml. of 2000 million *Salm. typhi* H suspension added to each dilution. The tubes were incubated for 2 hr. at 56° and read with × 10·5 magnification. Agglutination tests were made at intervals over a period of 30 days from the time of injection of the antigen; at daily intervals for 14 days following the injection of antigen and thereafter every 48 hr. or 72 hr.

Leucocyte counts.—Total leucocyte and differential white counts were carried out at the same time as the blood was withdrawn for agglutination tests.

RESULTS

Relation of Time of Injection of Antigen to Nitrogen Mustard Course on Antibody Production

Table II shows the total white cell counts and times of appearance of antibody and attainment of maximum titre as well as indicating the treatment of the various groups of animals.

It will be seen that the types of response obtained can be divided into two main categories:

(*a*) Groups in which the antigen was injected before the nitrogen mustard.

(*b*) Groups in which the antigen was injected at the beginning of the nitrogen mustard course or later during the course or after the course.

Groups in which antigen was injected at least 24 hr. before the beginning of the nitrogen mustard course (Groups II, III and IV)

It will be seen from Table II that the animals in Groups II, III and IV showed no delay or only 24 hr. delay in the appearance of antibody compared with the controls (Group I); the total leucocyte counts were normal at the time of injection of antigen and rose in the following 24 hr.; there was a long delay between the

TABLE II.—*Treatment of Animals in Groups I–VII, Total Leucocyte Counts at Time of Injection of Antigen, 24 hr. Later and at Times of Appearance of Antibody and Attainment of Maximum Titre*

Titres are also shown at time of appearance and at maximum titre.

	Total leucocyte counts in 100's/c. mm. at				Agglutination titres	
	Time of injection of antigen	24 hr. after antigen	Appearance of antibody	Time of maximum titre	Time of appearance*	Time of maximum titre*
Group I—						
Antigen only given . .	10·4	12·2	10·0	9·3	3/25	5/1600
	9·8	11·4	8·8	9·2	3/25	5/1600
	11·2	12·2	10·7	11·2	3/25	5/1600
Group II—						
Antigen given 72 hr. before	10·8	14·1	10·2	2·3	3/25	5/1600
N.M. course	9·6	15·6	11·2	4·2	3/100	5/800
	10·6	12·6	8·6	2·8	3/50	5/800
Group III—						
Antigen given 48 hr. before	9·8	14·4	6·6	10·8	3/25	21/3200
N.M. course	8·4	10·8	5·8	10·0	3/25	21/800
	10·4	12·8	7·2	9·5	3/25	19/800
Group IV—						
Antigen given 24 hr. before	10·4	20·4	1·2	11·0	4/25	17/3200
N.M. course	10·8	13·0	1·7	9·8	4/25	15/1600
	9·6	12·4	1·1	10·2	4/25	17/1600
Group V—						
Antigen given at beginning	10·4	7·7	4·3	11·2	7/25	16/1600
of N.M. course	10·6	9·8	3·1	9·2	6/25	12/800
	9·7	8·2	2·7	10·2	6/25	14/800
Group VI—						
Antigen given midway	5·0	1·5	4·8	6·1	5/25	8/800
through N.M. course	4·2	2·2	4·2	7·3	6/25	9/200
	6·1	3·2	6·3	10·6	7/25	10/200
Group VII—						
Antigen given 24 hr. after	1·4	1·2	10·4	9·6	8/25	11/1600
N.M. course	1·8	1·6	10·7	12·0	8/25	11/1600
	1·3	1·0	9·6	9·5	8/25	11/800

* Numerator = day following antigen. Denominator = reciprocal of titre on that day; *e.g.*, 3/25 = third day after antigen, titre = 1/25.

appearance of antibody and the attainment of maximum titre in Groups III and IV.

In Groups II, III and IV antibody production has begun before the effect of the nitrogen mustard has occurred (using as an indication of the effect of nitrogen mustard the behaviour of the circulating leucocytes) and having begun appears to continue even when the effects of the nitrogen mustard are manifest, notably in Group II. Maximum titre is not attained in Groups III and IV until the animal has recovered, the white cell counts having returned to normal in the meantime.

Groups in which antigen was injected at the beginning of the nitrogen mustard course, or midway during the course, or 24 hr. after the course

The animals of Groups V, VI and VII show a delay of 2–3 days at least in the appearance of antibodies, actually 5 days in the case of Group VII compared with the controls (Group I); the total leucocyte counts are either normal (Group V) or depressed (Groups VI and VII) at the time of injecting antigen and they did not rise 24 hr. later; there is less delay in the attainment of maximum titre after the appearance of antibody than in the case of Groups III and IV. These points are shown forth in Table II.

Further it will be seen that there is a gradation of response from Groups III to VII typified by a lengthening of the time interval between the injection of antigen and the appearance of antibody and a shortening of the interval between the appearance of antibody and the attainment of maximum titre.

Relation of Leucocyte Counts to Antibody Production

In all the animals which received a course of nitrogen mustard the total leucocyte counts fell to a minimum 48 hr. or 96 hr. after the end of the course and recovered in the following 7 days or so.

It has been noted that there is either no delay or a minimal delay in the appearance of antibody in those animals in which the total leucocyte counts were normal at the time of injection of antigen and for at least 24 hr. later (Groups II, III and IV) and a maximal delay in those animals in which the total leucocyte counts were lowest at the time of injection of antigen (Group VII). It is not suggested that there is a direct causal relationship between the behaviour of the total leucocyte counts and the production of antibody, probably both are manifestations of the toxic effects of the nitrogen mustard without their being causally connected but the possibility exists that there is a connection between the behaviour of the polymorphonuclear leucocyte count and the production of antibody. The polymorphonuclears are well established phagocytes and Sabin (1939) and Smith (1956) among many authors have shown that these cells carry soluble proteins and bacteria.

Since the administration of nitrogen mustard causes a granulo-cytopenia of a severe degree, the virtual absence of a system of phagocytes may well in part account for the delay in appearance of antibody since there may be a delay in the phagocytosis and therefore the breakdown of antigen.

Table III shows the correlation between the time of injection of antigen, the polymorphonuclear counts and the time of appearance of antibody and attainment of maximum titre.

The following points may be seen from Table III.—In the case of Groups II, III and IV the polymorphonuclear counts were normal at the time of injection of the antigen and for at least 24 hr. later and there is either no delay or only a minimal delay in the appearance of antibody. It is suggested that in these groups of animals breakdown of the antigen has occurred with little or no delay with a resultant normal time of appearance of the antibody, the long delay in Groups III and IV before the attainment of maximum titre being due either to delay in the synthesis of antibody or to delay in complete breakdown of the antigen as a result of the effects of the nitrogen mustard.

TABLE III.—*Relation between Polymorphonuclear Counts at Time of Injection of* Salm. typhi *H and 24 hr. after, also shows Time of Appearance of Antibody and of Attainment of Maximum Titre*

		Polymorphonuclear counts in 1000's/c. mm.. at		Agglutinins	
		Time of antigen	24 hr. after antigen	Time of appearance (days)	Time of max. titre (days)
Group I	Antigen only given	4·7	7·0	3	5
		4·3	6·9	3	5
		4·7	6·3	3	5
Group II	Antigen 72 hr. before N.M. course	4·4	7·1	3	5
		3·6	7·4	3	5
		4·7	6·9	3	6
Group III	Antigen 48 hr. before N.M. course	4·0	9·4	3	21
		3·7	3·5	3	21
		4·4	6·6	3	19
Group IV	Antigen 24 hr. before N.M. course	4·8	12·6	4	17
		4·4	6·5	4	15
		4·5	7·4	4	17
Group V	Antigen at beginning of N.M. course	4·8	4·0	7	16
		5·1	5·8	6	12
		4·5	4·2	6	14
Group VI	Antigen midway through N.M. course	2·6	0·2	5	8
		2·0	0·6	6	9
		2·9	0·7	7	10
Group VII	Antigen 24 hr. after N.M. course	0·1	0·1	8	11
		0·3	0·3	8	11
		0·2	0·08	8	11

In the case of Groups V, VI and VII the polymorphonuclear counts were either normal or reduced at the time of injection of antigen and fell in the following 24 hr. (except in the case of one animal of Group V) and there is a delay in the time of appearance of antibody; this delay in production of antibody would be, according to the suggestion just made, due to a delay in the breakdown of antigen as a result of the relative absence of the polymorphonuclear leucocytes, and also possibly due to an interference with the synthesis of antibody.

DISCUSSION

The results of the experiments show that the effects of nitrogen mustard on the antibody response of rabbits injected with 100 million *Salm. typhi* H intravenously depend on the time at which the antigen is injected with relation to the course of nitrogen mustard.

Spurr (1947) showed that when blood was taken at comparable times from animals injected with nitrogen mustard and normal animals, both groups having been injected with typhoid vaccine intramuscularly, the titres of the nitrogen mustard group were lower than those of the normal animals, but the recordings were taken at weekly intervals, so it is possible that a late rise in titre may have been missed by this method.

In the present study it would appear that a delay in appearance of maximal

titre is a constant feature in those animals in which antigen was injected 48 hr. or 24 hr. before the administration of nitrogen mustard but that a high titre was eventually reached. This finding prompts the question, " What is happening to the antigen and antibody production during the time at which the animal is under the effects of nitrogen mustard?"

The fact that normal or high titres can be obtained when the animals recover suggests that the antigen is not lost from the body during the time of maximal toxicity of the nitrogen mustard. The question arises as to whether the antigen is broken down during this stage or not, i.e., whether there is a pool of antigenic determinants as templates available but that they cannot be used due to the toxic effects of the nitrogen mustard on the animal.

There is no doubt that in the case of the animals injected 48 hr. or 24 hr. before the beginning of the nitrogen mustard course, antibody production had begun before the effects of the nitrogen mustard were maximal and indeed was continued during the phase of maximal injury. Marshall and White (1950) have shown that plasma cells are resistant to the effects of nitrogen mustard and X-rays and Benecerraf (1954) has shown that, under the influence of nitrogen mustard, although reticular and other cells are able to carry on phagocytosis, they are unable to divide and these findings would explain the low production of antibody obtained in the present study.

If one accepts Burnet's hypothesis (Burnet and Fenner, 1949 ; Burnet, 1956), namely, that once the antibody production mechanism has been initiated, the ability to produce antibodies is passed on to the progeny of the antibody producing cells (the cells of the plasma series), it is possible to see that as soon as the effects of the nitrogen mustard (which prevent mitosis occurring *inter alia*) have worn off, much more antibody will be produced by the progeny of the dividing antibody producing cells.

As regards the possibility of the slowing of the breakdown of antigen Schwab *et al.* (1950) have shown that there is a delay in the disappearance of bovine gamma globulin from the circulation of rabbits treated with nitrogen mustard or X-rays. Whether this delay is due to an effect on the R.E. cells is not known although Benecerraf *et al.* (1954) have shown that there is some effect on the phagocytic power of R.E. cells of nitrogen mustard treated rabbits when the cells have been blocked previously with carbon particles—he ascribes the loss of phagocytosis to the inability of the cells to divide.

It is possible however that the removal of the polymorphonuclear cells from the circulation by the action of nitrogen mustard may result in a slowing down of phagocytosis of the antigenic material.

When one considers what happens when the nitrogen mustard course precedes the injection of antigen, it is clear that there is a delay of several days before antibody appears and that antibody appears after the animal has begun to recover from the effects of the nitrogen mustard. In these animals there is not the long delay between appearance of antibody and attainment of maximal titre. There appear to be three possible reasons for this observed fact :

(1) That there is delay in the breakdown of antigen due to nitrogen mustard interfering with metabolic activity ;

(2) that there is delay in the synthesis of antibody following the breakdown of antigen ;

(3) that there is a combination of (1) and (2).

It is not possible to state which of the mechanisms is active although further experiments to be reported show that there appears to be a delay in the breakdown of antigen—the method used being one of the transfer of splenic tissue from donor animals injected with nitrogen mustard and antigen and later challenge of the recipients with the antigen.

SUMMARY

The effects of nitrogen mustard on the antibody response of rabbits injected with 100 million *Salm. typhi* H are related to the time at which the antigen is injected with respect to the course of nitrogen mustard.

The longest delay in appearance of antibody occurs when the antigen is injected following the nitrogen mustard; there is no delay when the antigen is injected at least 48 hr. before the beginning of the nitrogen mustard course.

In most cases maximum titre is attained when the animals have recovered from the effects of the nitrogen mustard.

The possible role of the polymorphonuclear leucocytes in the immune mechanism is discussed.

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