PARALLEL STUDIES OF COMPLEMENT AND BLOOD COAGULATION

XIV. IN TUBERCULIN SHOCK

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The tuberculin reaction is the classic example usually cited to illustrate the differences between acute anaphylactic shock and delayed antibacterial hypersensitivity. The most obvious difference between these two general forms of hypersensitivity is a temporal one, the tuberculin reaction taking much longer to develop than anaphylactic symptoms and in non-fatal cases persisting for a longer period. Furthermore anaphylactic sensitivity, which depends upon humoral antibody, can be transferred passively by the injection of serum from sensitized subjects, whereas this is not possible with tuberculin sensitivity. Normal animals may, however, be sensitized to tuberculin through transfer of white blood cells from tuberculous individuals of the same species (1, 2). Other points of difference have been discussed in innumerable reviews on this subject, one which has intrigued and puzzled investigators as long as immunology has existed as a science.

In our preceding paper in this series (3) were presented the results of a parallel study of the alterations in complement titre and plasma coagulability observed in groups of anaphylactic guinea pigs; the present paper will describe the results of a similar experiment in tuberculous guinea pigs following the intraperitoneal or intracutaneous injection of tuberculin. The two sets of experimental observations will be considered in relation to each other.

EXPERIMENTAL METHODS

Treatment of Guinea Pigs

Experiment 1:

On Sept. 5, 1950, forty guinea pigs, twenty male and twenty female, were injected intraperitoneally with 2.5 or 5 mg. of live, bovine type, tubercle bacilli of the moderately virulent strain No. 110, which is used in our routine production of tuberculin for cattle testing. In the late afternoon of Nov. 6, 8, 13 and 21, varying numbers of the surviving animals, a total of 27, were injected by the same route with 0.2 to 1.0 ml. of bovine type tuberculin, S25, prepared from the same culture strain. A total of 15 normal guinea pigs were injected with 0.2 to 0.5 ml. tuberculin on the same dates to serve as controls.

Two of the controls and three tuberculous animals were bled only once, at approximately 18 hrs. after the injection of tuberculin. The remainder were bled twice, at six hrs. before and 18 hrs. after tuberculin injection. Twelve normals that had not been injected with tuberculin were bled at the same time.

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All bleedings were made from the heart. The blood was divided into two parts, one being oxalated, the other allowed to clot.

Experiment 2:

On Nov. 19, 1953, twelve white male guinea pigs weighing 600 to 800 gms. were injected intraperitoneally with 20 mg. (moist weight) of a live culture of BCG, strain No. 346. On Dec. 16 five of these animals were injected by the intraperitoneal route with 0.5 ml. of 10% human type tuberculin (lot No. 14 [9]). The remaining six guinea pigs were injected intradermally with 0.1 ml. of the same dilution of tuberculin in three or six sites (three animals each). The skin reactions were read after 24 and 48 hrs. The intraperitoneally-injected guinea pigs were bled before the injection of tuberculin, one hr. later, and again after 48 hrs.; the intradermally-injected ones were bled but once, that is at 48 hrs.

A second tuberculin test was made on the ten surviving guinea pigs on Jan. 7, 1954; 1.0 ml. of 10% human type tuberculin was injected intraperitoneally. Five were bled five hrs. after tuberculin injection, the remaining four after 24 hrs., one animal having died in this interval. Two hours later the nine surviving guinea pigs were killed and examined to ascertain the degree of tuberculous involvement and the severity of the reaction to tuberculin.

Complement Titrations

Each serum was titrated to determine the amount required to produce 50% haemolysis of the standard volume of maximally sensitized, sheep-red-cell suspension. The haemolytic titre was expressed in 50% haemolytic units per ml. The titres of the four major complement components were determined with test reagents prepared from guinea pig complement, each reagent lacking the component for which the test was being made, but containing the other three.

Coagulation Tests

The coagulation tests were made by the one-stage method of Quick with undiluted plasma (4) and by a modified two-stage method with plasma diluted 1:25 (5, 6) which has been employed in our previous experiments.

EXPERIMENTAL RESULTS

Experiment 1:

This first series of guinea pigs, injected with a moderately virulent strain of bovine type tubercle bacillus, proved to be very sensitive to the intraperitoneal injection of tuberculin. Both of the animals given 1.0 ml. of tuberculin, nine of the 18 given 0.4 or 0.5 ml., and three of the seven injected with 0.2 ml. were found dead the following morning, that is only 13 of the 27 injected survived for 18 hrs. All animals autopsied showed extensive lesions.

Complement Titrations

The serum complement titres of the pre and post-injection bleedings from 10 control and the 13 surviving tuberculous guinea pigs are given in

Table 1. The injection of tuberculin had no appreciable effect on the whole complement titres of the control animals, nor was the titre of the individual complement components altered; the mean titres of first, second, third, and fourth components were 3442, 1705, 2175, and 8333 units per ml. respectively before tuberculin injection, and 3799, 1723, 2200, and 9999 units per ml. 18 hrs. afterwards.

TABLE I

CLOTTING TIME OF PLASMA AND COMPLEMENT TITRES OF SERUM OF CONTROL AND TUBERCULOUS GUINEA PIGS BEFORE AND AFTER TUBERCULIN INJECTION

No. of Guinea Pig	Group	Tuber-culin	Complement Titre (units/ml.)		Coagulation time (sec.)					
					Quick Method		2-Stage Method			
							Stage-I		Stage-II	
		ml.	1st. bl.	2nd. bl.	1st. bl.	2nd. bl.	1st. bl.	2nd bl..	1st. bl.	2nd. bl.
723	Norm.	0.5	1240	1160	29	26	189	157	84	71
724		0.5	1530	1390	29	27	208	206	95	93
798		0.4	1310	1280	29	26	144	149	42	57
799		0.4	1390	1480	26	27	143	147	44	57
800		0.4	1110	1390	24	26	149	146	45	42
996		0.2	1670	2000	23	29	142	150	54	49
999		0.2	1680	1370	24	21	175	150	34	32
998		0.2	1610	1470	29	29	162	163	49	59
997		0.2	1670	1610	29	26	212	192	57	77
995		0.2	1670	1280	24	29	164	184	36	65
878	Tbc.	0.4	1280	833	29	44	197	280	119	290
873		0.4	1390	693	29	41	211	306	113	408
883		0.4	1190	714	26	45	205	308	98	361
884		0.4	1560	454	29	49	159	240	41	213
886		0.4	1280	862	27	41	151	230	41	229
877		0.4	1470	999	26	45	140	254	36	342
894		0.2	1670	1370	25	41	135	250	36	196
892		0.2	1670	1560	24	26	167	184	42	72
993		0.2	1670	1390	30	47	155	238	41	167
887		0.2	1560	1320	27	37	179	202	58	116
Mean	Norm.		1488	1443	26.6	26.6	168.8	164.4	54.1	60.2
St. dev.			194	237	2.5	2.2	25.1	20.5	19.0	16.6
Mean	Tbc.		1474	1019	27.2	41.6	169.9	249.2	62.5	239.4
St. dev.			177	149	1.9	6.1	25.6	38.3	31.9	103.6

The complement titres of the tuberculous animals compared closely with those of the controls before the injection of tuberculin. The sera collected 18 hrs. after the injection of 0.4 ml. tuberculin had considerably lower complement titres than initially; the mean titre for these six animals fell from 1362 units per ml. (standard deviation 75) to 759 units per ml. (st. dev. 186), a highly significant difference (P less than 0.001). The mean titres of the two bleedings from the four tuberculous animals given the 0.2 ml. dose of tuberculin fell within the normal range; 1433 and 1410 units per ml. This sug-

gested that the reduction in complement activity might be related directly to the amount of tuberculin injected.

Of the four major complement components, the first and second were somewhat decreased in the tuberculous animals injected with 0.4 ml. tuberculin. The mean first-component titres of these six guinea pigs were 3445 units per ml. before, and 2262 units per ml. after injection: the mean second-component titres of the two bleedings were 1237 and 1090 units per ml. respectively. The third-component titres were not sensibly affected, the mean values for the two bleedings from the group, being 2200 and 2100 units per ml. Some reduction in fourth-component titres was recorded in four of the six animals receiving 0.4 ml. tuberculin; before injection all had titres over 10,000 units per ml., afterwards their mean serum titre in this component was 6338 units per ml.

Coagulation Tests

In the first day's test, the two control and three surviving tuberculous guinea pigs were bled only once, that is 18 hrs. after the intraperitoneal injection of 0.2 to 0.4 ml. tuberculin. The prothrombin time of the diluted plasma as determined by the Quick method was closely comparable for the control and experimental animals; mean 27 and 32 sec. respectively. Somewhat greater differences were noted in the values obtained by the two-stage method with diluted plasma. The mean stage-I clotting time (without added fibrinogen) was 191 sec. for the controls, 218 sec. for the tuberculous; the mean stage-II clotting time (determined in the presence of added fibrinogen) was 85 sec. for the former, 144 sec. for the latter.

In the next three tests, all animals were bled before as well as 18 hrs. after tuberculin injection since it seemed possible that even before tuberculin injection the plasma of the tuberculous animals might show delayed coagulability. As indicated in Table I, however, such was not the case: the two-stage clotting times of the tuberculous and control guinea pigs were closely comparable before tuberculin injection. Furthermore, the injection of tuberculin did not induce any prolongation of plasma clotting time in the controls, indeed in several instances the clotting time of the post-injection bleedings was shorter than that of the first.

In the tuberculous animals, however, the increase in the Quick prothrombin time ranged from 2 to 20 sec., in stage-I clotting time from 17 to 115 sec. and in stage-II clotting time from 30 to 306 sec. The man Quick prothrombin time and the mean stage-I clotting time of the second bleeding from the tuberculous guinea pigs were both significantly longer than those of the first bleeding from the same animals or of the second bleeding from the tuberculin-injected controls (P less than 0.001). The mean stage-II clotting time of the tuberculous animals, although significantly longer than that of the controls (P more than 0.001, less than 0.01), was not significantly longer than that of the first bleeding from this group (P greater than 0.05).

In the present experiment, as has been our general finding, the stage-II

clotting times of individual specimens of diluted plasma were about 100 sec. shorter on the average than the stage-I clotting times of the same. In other words, the addition of fibrinogen reduced the total time required for coagulation by about 55 sec. if correction is made for the preliminary period of 45 sec. Allowed for conversion before removal of the sample for the stage - II test. The stage-II clotting times of the first bleeding from the tuberculous guinea pigs ranged from 78 to 125 sec. (mean 107 sec.) shorter than their stage-I clotting times. The addition of fibrinogen in stage-II had a much lesser effect on the clotting time of plasma of the second bleeding from these animals; for three, Nos. 873, 883, and 877, the stage-II clotting times were longer than the stage-I clotting times even when corrected for the 45 sec. conversion period. This suggested that a decrease in prothrombin rather than fibrinogen was responsible for the prolongation in clotting time. In view of our more recent findings, however, it seems possible that a decrease in Ac-globulin may also have occurred.

Experiment 2:

In this experiment a small number of BCG-sensitized guinea pigs were tested for tuberculin sensitivity 27 and 49 days after inoculation with these organisms. In the first tuberculin test, half of the animals were injected intraperitoneally, half intradermally. When the skin tests were read at 24 and 48 hrs. all showed moderate to marked reactions. In the second test, the eight surviving guinea pigs were sacrificed about 31 hrs. after tuberculin injection; these eight and the two that had died previously were autopsied and examined for tuberculous lesions. These were localized in the tissues and organs of the abdominal cavity, the omentum being involved in nine, the peritoneum in seven, the testicle or epididymis in six, the liver in four and the spleen in three.

An attempt was made to assess the degree of the tuberculin reaction on the basis of the severity of the inflammation in the peritoneum, the amount and nature of the resultant exudate, and the presence of a haemorrhagic zone around the necrotic lesions in the liver and spleen. According to these criteria, the reactions in the ten animals were grouped under four, not-very-well-defined headings: slight to moderate (four), moderate (three), moderate to fairly pronounced (two), and pronounced (one).

The serum specimens taken from the animals before the first injection of tuberculin were tested for complement-fixing activity with an antigen prepared from acetone-extracted bovine-type tubercle bacilli. All but two had moderately high titres, 1:200 or over. The two animals with low complement-fixing titres showed moderately marked intradermal reactions to tuberculin. None showed more than weak fixation with the bovine type tuberculin as antigen.

Complement Titrations

The pre-injection bleedings and those taken one hour later from the five intraperitoneally-injected, BCG-infected guinea pigs had complement titres comparable to those of the normal group (Table II). Two of the four surviv-

TABLE II

THE EFFECT OF THE INTRAPERITONEAL OR INTRADERMAL INJECTION OF TUBERCULIN ON COMPLEMENT TITRE OF THE SERUM AND THE COAGULATIVE ACTIVITY OF THE PLASMA OF BCG-INFECTED GUINEA PIGS (FIRST TUBERCULIN TEST)

Group	Tuberculin treatment		Bleeding	Number tested	Clotting time (sec.)				Complement titre units/ml.	
	Route	Amount ml.			Stage-I		Stage-II			
					Mean	St. dev.	Mean	St. dev.	Mean	St. dev.
Normal		0	Pre.	3	219	7	70	4	1547	71
			1 hr.	3	237	21	76	4	1350	50
			48 hrs.	3	224	22	85	9	1590	296
BCG-infect.	ip.	0.1	Pre.	5	212	18	107	14	1642	187
		0.1	1 hr.	5	224	27	101	49	1488	171
		0.1	48 hrs.	4	180	31	116	39	1185*	260
BCG-infect.	id.	0.0075	Pre.	3	206	19	99	34	1463	127
			48 hrs.	3	163	16	88	12	1850	81
BCG-infect.	id.	0.0150	Pre.	3	193	23	81	10	1583	127
			48 hrs.	3	165	17	107	19	1510	138

*Significantly different from the initial value: (P more than 0.001, less than 0.01).
ip. = intraperitoneal; id. = intradermal.

ing animals in this group had lower complement titres 48 hrs. after tuberculin injection than they had initially or one hr. after injection. The intradermally-injected tuberculous guinea pigs had titres within the 'normal' range at 48 hrs.

In the second series of tuberculin tests, two normal and five tuberculous guinea pigs were bled five hrs. after the intraperitoneal injection of 1.0 ml. of tuberculin and their complement titres compared with those of two control untreated animals (Table III). Four of BCG-infected guinea pigs had lower titres than those of the four controls. Sera collected at 24 hrs. from two normals injected with tuberculin, two untreated controls, and the four surviving tuberculous animals were likewise titrated for complement content. Three of the four tuberculous animals had definitely depressed titres, in the fourth the titre was similar to those of the controls. In the two tuberculous guinea pigs that showed no fall in complement titre, the response to tuberculin had been classified as slight to moderate, that is in the lowest category. Two other animals showing the same general degree of tissue response had depressed complement titres.

Coagulation Tests

The stage-I clotting times of the plasma of the BCG-infected guinea pigs

TABLE III

THE EFFECT OF THE INTRAPERITONEAL INJECTION OF TUBERCULIN ON THE COMPLEMENT TITRES OF THE SERUM OF NORMAL AND BCG-INFECTED GUINEA PIGS (SECOND TUBERCULIN TEST)

Group	Tag No. of Guinea Pig	Complement titre (units/ml.) Bleeding			Tuberculin reaction
		Pre	5 hrs.	24 hrs.	
Control	1	1250	1110	x	None
	2	1320	1250	x	None
	3	1470	x	1320	None
	4	1610	x	1390	None
BCG-infected	330	1560	746	x	Moderate
	328	1430	714	x	Moderate
	327	1790	961	x	Moderate
	333	1470	758	x	Slight to moderate
	335	1720	1280	x	Slight to moderate
	324	1850	x	587	Pronounced
	325	1670	x	862	Slight to moderate
	332	1510	x	1510	Slight to moderate
	342	1560	x	714	Moderate to pronounced
Control	Mean	1413	1180	1355	
BCG-infected	Mean	1618	892*	918	

*Significantly lower than mean complement titre for initial bleedings from the same animals.

before injection of tuberculin were comparable to those of normal animals, whereas four of the ten had stage-II clotting times somewhat longer than normal (Table II). Neither the stage-I nor the stage-II clotting times of the plasma was significantly prolonged after the intraperitoneal injection of tuberculin. The stage-I clotting times of the 48 hr. bleedings were comparable to or even definitely below the initial value; maximum decrease 70 sec. The stage-II clotting times on the other hand tended to increase slightly in eight of the guinea pigs: maximum increase 65 sec.; in one case it fell by 44 sec. Considered in relation to each other, these findings suggested that fibrinogen had increased in these tuberculous guinea pigs during the inflammatory response to tuber-

culin, as has been reported for other animals (7); whereas prothrombin, and possibly also Ac-globulin, was decreased in the majority of them. This response is the converse of that observed during acute anaphylactic shock in which fibrinogen tended to fall and prothrombin to be relatively much less affected (1).

Since tests of plasma coagulability were not made on the blood specimens collected during the second series of tuberculin tests of this group of animals, no information was obtained in regard to the coagulative behaviour during the early post-injection period.

DISCUSSION

In view of the fact that the time of development of symptoms is one of the major differences between anaphylactic and tuberculin shock, it was not surprising to find that complement decline was likewise delayed in its appearance. This raises the question of the location of the antigen-antibody reaction responsible for the uptake of serum complement in the latter instance. In anaphylatic shock it has been assumed that the loss in complement activity, demonstrable immediately after the intravenous injection of the shocking dose of antigen, is partially at least the result of combination of this antigen with humoral antibody. In tuberculin shock, since the drop in complement titre occurs much later, it may be thought to result from its fixation by complexes formed between circulating antibody and antigenic material liberated from the focus of infection as a secondary effect of the tuberculin reaction, circulating antibody showing little fixation of complement in the presence of tuberculin. In that this antibody may have no direct connection with tuberculous hypersensitivity *per se* (8), such a fall in complement titre would be related more closely to the secondary than to the primary reaction. It might be expected accordingly to continue as long as antigenic material was being liberated from the focus and a residium of antibody was still present in the serum. The fact that complement titres were still depressed 24 and 48 hrs. after tuberculin injection would seem to lend support to this hypothesis since it is known that complement is rapidly regenerated in the guinea pig unless there is extensive liver damage. In the surviving anaphylactic animals, complement activity was at normal levels by the following morning (1). In other words, the more prolonged depression of complement in the tuberculin-shocked animals suggested a continued inactivation of regenerated complement.

An additional or alternative source of complement inactivation may lie in the lysis of white blood cells during the protracted period of leucopenia that follows tuberculin injection (9). The observation of Stewart, Long, and Bradley (10) that in tuberculin and reinfection reactions in tuberculous animals, the reacting inflammatory cells appear to be themselves sensitized and in consequence undergo early death, was investigated further by Favour (11) and others of this group (12, 13). Their *in vitro* studies have indicated that complement and a sensitizing factor present in the serum or plasma of tuberculous

animals or patients, or slowly released from leucocytes of such individuals, are both involved in the lysis of white blood cells in the presence of tuberculoprotein (12, 13). The sensitizing substance appears to be a heat-labile globulin, remaining in fresh serum after complement has been removed by treatment with various antigen-antibody systems. Such de-complemented sera lack sensitizing activity but this may be restored by the addition of fresh normal serum which by itself has no sensitizing properties (12). Experiments with the haemagglutination test have shown that erythrocytes sensitized with tuberculin or extracts of tubercle bacilli are likewise lysed when complement is added (14, 15), a phenomenon which may apparently also occur *in vivo* (16).

The observation that the extent of the complement decline in our infected guinea pigs was greater the larger the volume of tuberculin injected would seem explainable on the basis of either of these suggested inactivation processes.

SUMMARY

The changes in serum complement and plasma coagulability after the injection of tuberculin were followed in two groups of guinea pigs, one infected with a moderately virulent strain of bovine tubercle bacillus, the other with BCG.

A large proportion of the animals in the first group died in less than 18 hours after the intraperitoneal injection of the tuberculin but those that survived for this period showed a definite fall in complement titre and an appreciable prolongation of plasma clotting time. The BCG-infected guinea pigs, although exhibiting moderate to marked reactions in intradermal tuberculin tests and a very considerable decrease in serum complement activity, displayed relatively less alteration in plasma clotting time following tuberculin injection by the intraperitoneal route than the first group. No direct relationship was therefore observed between changes in plasma coagulability and complement titres in these tuberculous animals after tuberculin injection.

Blood collected one hour after tuberculin injection showed no decline in complement titre, whereas in our previous studies of the behaviour of anaphylactic guinea pigs, a marked fall in complement was detectable almost immediately after the intracardial or intravenous injection of homologous antigen. Conversely, complement titres had returned to normal in 24 hours in the latter series, whereas they were still depressed at 48 hours in the tuberculin-injected tuberculous animals.

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COMING MEETINGS OF VETERINARY MEDICAL ASSOCIATIONS

Association	Place	Dates
College of Veterinary Surgeons of the province of Quebec	Montreal, Que.	Aug. 13-14, 1954.
American Veterinary Medical Association	Seattle, Wash.	Aug. 23-26, 1954.
Canadian Veterinary Medical Association	Ottawa, Ont.	Aug. 30-31, Sept. 1, 1954.

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