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Granulomatous Hypersensitivity to Trehalose-6,6'-Dimycolate (Cord Factor) in Mice Infected with BCG

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Cord factor in the form of emulsion is unable to sensitize mice to react with a more extensive granulomatous response to a subsequent challenge with the same substance. Mice infected with BCG bacilli are sensitized to cord factor. Such animals react to administration of cord factor with a very extensive granulomatous response, much stronger than normal ones. This hypersensitivity seems to be specific and is distinct from delayed hypersensitivity to PPD. PPD, administered to mice sensitized with BCG bacilli, has no effect on the granulomatous response. Our findings and their importance are discussed.

The lungs of rabbits sensitized with heat-killed, oil-treated BCG organisms react with an increased and accelerated granulomatous response after an intravenous administration of BCG cells in saline (14). A similar reaction can be induced in the lungs of mice by intravenous injections of large numbers of mycobacteria. The magnitude of the response is related to the amounts of mycobacteria injected (17, 18). There is also a marked macrophage accumulation in the lungs of mice after intravenous vaccination of these animals with oil-treated mycobacterial cell walls (1). It has recently been shown that trehalose-6,6'-dimycolate (cord factor), a glycolipid extractable from mycobacteria, is able to induce a granulomatous response in the lungs of mice after an intravenous injection of as little as 1 to 5 μg. The cellular composition of the tubercules induced by cord factor is indistinguishable from that caused by living BCG cells. In both, the granulomas are composed of epithelioid cells, macrophages, and lymphocytes. Cord factor injected into the footpads of mice caused histological changes similar to those which resulted after injections of living BCG cells. Both cord factor and living BCG cells induced formation of granulomas, marked hyperplasia of the lymphoid tissue in the paracortical zone, and accumulations of macrophages in the draining lymph nodes. Because no cellular reaction was evident in organs such as liver, spleen, and lungs, it was possible to show that the increased antibody response to an unrelated antigen, which was observed in such animals, is of a local character, dependent on the abovementioned cellular reaction (2-4). Recently, it became evident that cord factor suppresses the development of urethane-induced tumors in the lungs of mice to an extent similar to that induced by living BCG cells, inhibition apparently due to the granulomatous reaction caused locally in the lungs by cord factor (5).

It was shown in this laboratory that repeated intravenous injections of cord factor into mice induced a more extensive granulomatous response than did a single injection of the same total amount of cord factor (2). This observation raised the question of whether the more extensive cellular response is an expression of some kind of sensitization caused by cord factor and whether cord factor plays a role in the increased tubercle formation in mice sensitized with BCG cells. As will be seen from the data in this paper (i) cord factor alone failed to sensitize mice to react with a more extensive granulomatous response to a subsequent injection of the substance, (ii) BCG-infected mice responded with a strong granulomatous response to cord factor (this sensitization seems to be specific) and (iii) BCG-infected mice do not respond with an increased and accelerated tubercle formation to PPD.

MATERIALS AND METHODS

Animals. Albino mice of a local strain and mice of strain ICR provided by the Weizman Institute in Rehovot, Israel, were used throughout all the experiments.

Mycobacterial fractions and other materials. Cord factor from strains *Mycobacterium* var. *hominis* Peurois and *Mycobacterium bovis* strain BCG were used. Both types of cord

factor as well as trehalose-6,6'-dipalmitate were prepared in the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France, by E. Vilkas and R. Toubiana, respectively. Dimycoloyl sucrose was prepared by F. Asselineau, Centre de Recherche de Biochimie et de Genetique Cellulaire, Toulouse, France. The suspension of living BCG was prepared from 6- to 8-day cultures of the bacilli grown in Dubos broth base (Difco).

Preparation of emulsions and other materials. The emulsions of cord factors and sucrose-6,6'-dimycolate were prepared as previously described (2) by sonic treatment in an ultrasonic oscillator (Measuring and Scientific Equipment Ltd., London, England).

In the preparation of the emulsion of trehalose-6,6'-dipalmitate, a glass-Teflon homogenizer was used instead of the ultrasonic oscillator. The histological preparations of the lungs were prepared as described previously (2). After staining with hematoxylin and eosin, the number of the granulomas were counted in the sections of the lungs, and the surface of the sections was measured.

Statistical evaluation of results. The nonparametric rank test of Wilcoxon has been used (16).

RESULTS

Kinetics of granuloma induction by cord factor. The observation that repeated injections of small amounts of cord factor induce a more extensive granlomatous response in the lungs of mice than a single injection of the same total amount was based on an arbitrary evaluation of the cellular reaction in which the surface of the lung sections examined was not measured and the time of development of granulomas was not taken into consideration (2). We thought that the experiments related to the above observations should be repeated after an investigation of the time factor in the development of the granulomas.

Four groups of mice (local strain), five mice in each group, were injected intravenously with 20 μg of cord factor (Peurois). The mice were killed at 1, 3, 7, and 14 days after injection of cord factor. The presence of granulomas was evaluated with a microscope by counting the number of granulomas in all sections present in the histological preparation of the lungs. The surface of the sections was measured, and the number of granulomas per 100 mm² was established. The granulomas were already present after 24 h (Table 1). They increased in number and size after 3 and 7 days. It was apparent that, after 7 days, their number decreased, reaching at 14 days a level close to that reached after 1 day. The differences between groups 1, 2, 3, and 4 are statistically significant. Although there was a slight increase in the average number of granulomas after 7 days compared with that after 3 days, the differences were not statistically significant.

TABLE 1. *Time relationship between administration of cord factor and the granulomatous response in the lungs of mice*[a]

Group no.	Time interval (days)	No. of granulomas per 100 mm² in individual mice					Avg per 100 mm²
		1	2	3	4	5	
1	1	10	11	11	15	54	20.2[b]
2	3	104	107	127	141	142	124.2
3	7	53	107	122	182	219	136.6
4	14	14	22	26	32	62	31.2

[a] The amount of cord factor (Peurois) administered intravenously was 20 μg in emulsion containing 1% Bayol F and 1% Tween 80 in saline in a volume of 0.2 ml.

[b] The differences in the average numbers of granulomas between groups 1, 2, 3, and 4 are statistically significant. The respective values of P are 0.005 and 0.01. The difference between groups 2 and 3 is not statistically significant.

The effect of repeated injections of cord factor on the granulomatous response. The experiment was carried out on mice, strain ICR, which in a separate study showed a very high sensitivity to injections of relatively small quantities of cord factor. Groups of 10 mice were injected intravenously with 4 and 2 μg of cord factor (Peurois), and after an interval of 3 days the latter groups were challenged with the same quantity of cord factor from strain Peurois or from strain BCG. Four days after the second injection the mice were killed, and the granulomatous response was evaluated (Table 2). Although the group of mice which was injected twice with 2 μg of cord factor appeared to display a stronger response than the group injected with 4 μg, the differences are not statistically significant.

The results seem to indicate that cord factor is unable to sensitize the mice during 3 days, the time interval after which cord factor was administered the second time. We assumed that 3 days may be an insufficient length of time to sensitize the mice; therefore, an experiment has been carried out to test this assumption. Five groups of mice were injected with cord factor and challenged after 14 days with a second injection of a smaller amount of cord factor. The lungs of the mice were tested after 4 days. The results are presented in Table 3. It is clear that after 14 days the granulomatous response to the second injection is no greater than that after the first one. On

TABLE 2. *Granulomatous response in the lungs of mice after repeated injections of cord factor (cf)*[a]

Group no.	Treatment	No. of granulomas per 100 mm² in individual mice										Avg per 100 mm²
		1	2	3	4	5	6	7	8	9	10	
1	cf (Peurois), 4 μg; emulsion	115	200	220	151	199	148	154	67	89	94	143.7
2	cf (Peurois), 2 μg; cf (Peurois), 2 μg	290	167	204	102	171	119	205	180	120	184	174
3	Emulsion; cf (Peurois), 2 μg	155	49	94	74	97	106	237	149	9	127	109.7
4	cf (Peurois), 2 μg; emulsion	93	181	97	125	19	89	64	154	119	128	106.9
5	cf (Peurois), 2 μg; cf (BCG), 2 μg	164	147	135	141	211	101	103	193	136	107	143.8
6	Emulsion, 2 μg; cf (BCG), 2 μg	4	12	9	0	8	35	26	19	35	48	19.6

[a] The mice used were of strain ICR. The interval between two injections of cord factor or cord factor and emulsion was 3 days. The lungs of the mice were examined 4 days after the second injection. The emulsions used contained 1% Bayol F and 1% Tween 80 in saline. Volumes of injected materials were 0.2 ml.

TABLE 3. *Granulomatous response in the lungs of mice at an interval of 14 days between two injections of cord factor*[a]

Group no.	Treatment of mice[b]	No. of granulomas per 100 mm² in the lungs of individual mice										Avg per 100 mm²
		1	2	3	4	5	6	7	8	9	10	
1	cf (Peurois), 2 μg i.v.; emulsion i.v.	0	0	1	2	3	4	4	4	5	8	3.1
2	cf (Peurois), 2 μg i.v.; cf (Peurois), 1 μg i.v.	8	14	15	19	22	23	36	89	138		40.4
3	emulsion i.v.; cf (Peurois), 1 μg i.v.	35	35	37	48	68	76	78	89	106	175	74.7
4	cf (Peurois), 2 μg i.p.; emulsion i.v.	0	0	0	0	0	0	0	0	1	3	0.4
5	cf (Peurois), 2 μg i.p.; cf (Peurois), 1 μg i.v.	3	11	17	17	19	25	30	32	44	49	24.7

[a] The lungs of the mice were examined 4 days after the second injection.

[b] The mice used were of strain ICR. The difference between group 2 and groups 1 and 3 paired together is statistically significant, $P < 0.05$, as is the difference between group 5 and groups 4 and 3 paired together. Abbreviations: i.v., intravenous; i.p., intraperitoneal; cf, cord factor.

the contrary, after a second injection of cord factor, the response in the lungs was weaker. The number of granulomas induced by 1 μg of cord factor in mice of group 3 is almost three times that of group 5, in which the mice were injected intraperitoneally with 2 μg and subsequently challenged with 1 μg intravenously. This difference is statistically significant ($P < 0.02$). The number of granulomas in group 3 is also larger than in group 2 ($P < 0.05$), in which the mice were administered with cord factor intravenously two times.

The granulomatous response to killed BCG and PPD in mice sensitized with living BCG. The above results indicate clearly that although cord factor by itself induces a very strong granulomatous response even when injected in minute amounts, it does not enable the mice to react with a stronger cellular reaction to a subsequent administration. We assumed that the

form in which cord factor is administered to mice may be crucial for sensitization. To test this assumption it was necessary first to see whether the granulomatous response to dead BCG bacilli is stronger in mice sensitized with living BCG, and whether PPD has any effect on this reaction. To answer this question we took advantage of the observation made by us and others that living BCG bacilli injected intraperitoneally into mice cause a very weak granulomatous response in their lungs, which is in striking contrast to the very strong response after an intravenous injection (2, 17).

Groups of 10 mice were injected intraperitoneally with living BCG and challenged with dead BCG and PPD intravenously after 19 days. The lungs were examined 4 days later. The results are presented in Table 4. The granulomatous reaction in the sensitized mice was substantial in response to a subsequent injection of dead BCG bacilli. There was no observable change in response to injection of PPD.

Granulomatous response to cord factor in mice sensitized with living BCG. The above result suggested that the "accelerated tubercle" formation is due to a sensitizing component of the bacilli different from tuberculoprotein. As indicated above, it was assumed that cord factor would be the most plausible constituent. To test this, groups of 10 mice were injected intraperitoneally with a suspension of living BCG (about 1.2×10^7 cells). Nineteen days after administration of the bacilli the mice were injected with 2 μg of cord factor from strain Peurois or strain BCG. Control groups were injected intravenously with the respective cord factors only. Four days afterwards the mice were killed, and the granulomatous response in their lungs was evaluated. A group of 10 mice vaccinated by the intraperitoneal route with BCG was also injected intravanously with 50 μg of PPD, to test whether it plays a role in granuloma formation. It is apparent (Table 5) that the BCG injection sensitized the mouse to both kinds of cord factors. The number of granulomas per 100 mm^2 of lung sections in the BCG-vaccinated group increased from 11.3 to 35.5 after injection of cord factor (BCG). Injection of cord factor (BCG) alone induced 6.3 granulomas per 100 mm^2. The differences in the number of granulomas between the BCG-sensitized, cord factor (BCG)-challenged group (group 3) and groups 1 and 5 paired together are highly significant statistically ($P = 0.001$). The same is also true for the group challenged with cord factor from strain Peurois. On the other hand, in the group of mice sensitized with BCG and injected with 50 μg of PPD, no significant change in the number of granulomas was observed, confirming once more the previous result.

Specificity of sensitization to cord factor by BCG bacilli. The increased granulomatous response to the two kinds of cord factors of mice injected with BCG indicated that the infection causes sensitization to cord factor. This raised the question of its specificity. To answer this question, groups of 10 mice sensitized intraperitoneally with BCG were challenged 19 days after injection of the bacilli with trehalose-6,6′-dipalmitate, which

TABLE 4. *Granulomatous response in BCG-sensitized mice to challenge with killed BCG bacilli and PPD*[a]

Group no.	Treatment of mice[b]	No. of granulomas per 100 mm^2 in the lungs of individual mice										Avg per 100 mm^2
		1	2	3	4	5	6	7	8	9	10	
1	BCG	0	0	0	0	0	0	0	0	8	16	2.4
2	BCG; killed BCG, i.v.	0	2	6	9	21	34	42	50	78	85	32.7
3	Killed BCG, i.v.	0	0	0	0	0	0	0	0	0	9	0.9
4	BCG; PPD, 80 μg i.v.	0	0	0	0	0	4	5	6	7	49	7.1

[a] The suspension of living BCG, of an optical density of 70 Klett units (about 5×10^7 per milliliter was injected intraperitoneally in a volume of 0.2 ml. The suspension of heat-killed BCG bacilli of an optical density of 75 Klett units was injected intravenously in a volume of 0.2 ml, 19 days after the intraperitoneal administration of BCG. PPD in saline was also injected at the same time. The lungs of the mice were examined 4 days after the intravenous challenge.

[b] The mice used were of strain ICR. The difference between group 2 and groups 1 and 3 paired together is statistically significant, $P < 0.01$. The difference between groups 1 and 4 is statistically not significant, $P > 0.1$.

is also able to induce a granulomatous response in the lungs of mice after an intravenous injection (unpublished results). The results (Table 6) indicate quite clearly that the BCG-sensitized mice failed to react with an increased granulomatous response to injection of trehalose-6,6′-dipalmitate, a substance which is also granulomagenic but different chemically from cord factor. This experiment and the former have been repeated and extended. Besides trehalose dipalmitate, sucrose-6,6′-dimycolate was used. The latter substance, the lipid moiety of which has the same structure as cord factor, is also granulomagenic in mice (unpublished results). Groups of mice were injected intraperitoneally with living BCG bacilli and after 19 days were challenged intravenously with trehalose-6,6′-dipalmitate, sucrose-6,6′-dimycolate, and cord factor from BCG. The results are presented in Table 7. There is no doubt that a BCG infection sensitized the mice to cord factor. The granulomatous response in their lungs was about two times greater than in those injected with cord factor alone. The difference between group 3 and groups 1 and 2 is highly significant statistically ($P < 0.001$). Although the granulamatous response in the group of mice challenged with trehalose dipalmitate is stronger than in groups 1 and 4 paired together, the difference is not statistically significant ($P > 0.05$). On the other hand, it seems that the mice

TABLE 5. *Granulomatous response to cord factor in BCG sensitized mice*[a]

Group no.	Treatment of mice[b]	No. of granulomas per 100 mm² in individual mice										Avg per 100 mm²
		1	2	3	4	5	6	7	8	9	10	
1	BCG; emulsion	1	5	87	2	3	6	0	0	5	5	11.3
2	BCG; cf (Peurois), 2 μg	80	222	113	210	169	100	123	142	61	113	133.3
3	cf (Peurois), 2 μg	139	1	62	3	16	28	42	41	101	45	47.8
4	BCG; cf (BCG), 2 μg	39	28	59	45	48	20	43	25	27	21	35.5
5	cf (BCG), 2 μg	6	2	11	0	9	4	6	9	11	5	6.3
6	BCG; PPD, 50 μg	4	0	0	2	1						1.4

[a] The suspension of BCG, of an optical density of 80 Klett units, was injected intraperitoneally in a volume of 0.2 ml. Cord factors, emulsion, and PPD were injected intravenously 19 days after the intraperitoneal administration of BCG. The lungs of the mice were examined 4 days after the intravenous administration of the materials.

[b] The mice used were of strain ICR. The difference between group 2 and groups 1 and 3 paired together is statistically highly significant, $P < 0.01$, as is the difference between group 4 and groups 1 and 5, $P = 0.001$. The difference between group 6 and group 1 is not statistically significant.

TABLE 6. *Granulomatous response of BCG sensitized mice to trehalose-6,6-dipalmitate*

Group no.	Treatment of mice[a]	No. of granulomas per 100 mm² in the lungs of individual mice										Avg per 100 mm²
		1	2	3	4	5	6	7	8	9	10	
1	BCG; emulsion	0	5	0	0	0	2	0	23	6	16	5.2
2	Trehalose-6,6-dipalmitate, 20 μg	3	2	1	16	4	35	0	1	1	8	7.1
3	BCG; Trehalose-6,6-dipalmitate, 20 μg	7	6	18	3	9	13	32	6	42	7	14.3

[a] The BCG suspension was injected intraperitoneally, trehalose-6,6-dipalmitate intravenously. Other details as in legend to Table 3. The difference between group 3 and groups 1 and 2 paired together is statistically not significant.

TABLE 7. *Granulomatous response to cord factor (BCG), trehalose-6,6'-dipalmitate, and sucrose-6,6'-dimycolate in mice sensitized with living BCG*[a]

Group no.	Treatment of mice	No. of granulomas per 100 mm² in the lungs of individual mice										Avg per 100 mm²
		1	2	3	4	5	6	7	8	9	10	
1	BCG, i.p.; emulsion	0	0	0	0	0	2	4	8	13	34	6.1
2	cf (BCG), 2 μg i.v.	18	22	23	28	30	35	35	44	45	49	32.9
3	BCG i.p.; cf (BCG), 2 μg i.v.	56	73	77	80	89	95	102	108	114		88.2
4	Trehalose dipalmitate	0	0	0	2	2	3	3	5	5	16	3.6
5	BCG, i.p.; Trehalose dipalmitate, 20 μg i.v.	0	2	12	21	23	34	36	39	53	62	28.2
6	Sucrose dimycolate	0	0	0	0	0	1	1	3	4	7	1.6
7	BCG i.p.; sucrose dimycolate, 10 μg i.v.	4	4	9	11	16	20	22	23	48		17.4

[a] The suspension of living BCG, of an optical density of 50 Klett units, was injected intraperitoneally in a volume of 0.2 ml. The interval between the injection of BCG bacilli and the other materials was 19 days. The lungs of the mice were examined 4 days after the intravenous administration of cord factor, trehalose dipalmitate, and sucrose dimycolate.

[b] The mice used were of strain ICR. The difference between group 3 and groups 1 and 2 paired together is statistically highly significant, $P < 0.001$. The difference between group 7 and groups 1 and 6 is significant, $P < 0.05$; the difference between group 5 and groups 1 and 4 is not significant, $P > 0.05$.

infected with BCG are sensitized to sucrose-6,6'-dimycolate. The difference between group 7 and groups 1 and 6 paired together is statistically significant ($P < 0.05$).

DISCUSSION

It is clear from the present study that mice pretreated with an emulsion of cord factor do not respond with an increased granulomatous response to a repeated administration of the latter. Thus, our previous observation was not confirmed in the present experiments in which the kinetics of the development of the granulomas were considered and the results were evaluated in a quantitative way. It is of interest that, at an interval of 14 days between two injections of cord factor, the number of granulomas in the lungs is smaller than after one injection. One may speculate that, during a 14-day interval, antibodies have been formed which neutralize the second administration of cord factor. This is under investigation. Relevant to this assumption is the work of Kato (11), who showed that a complex of trehalose-6,6'-dimycolate and methylated bovine serum albumin induced in mice and rabbit antibodies which neutralize cord factor.

The present studies have shown that mice sensitized with living BCG cells responded with an intense tubercle formation subsequent to challenge with cord factor. Apparently, this response is an expression of some form of a hypersensitivity reaction, because it could be induced only with cord factors or whole organisms. The reaction to two other granulomagenic substances, which are similar in their chemical structure to cord factor, was much weaker. It is of interest that sucrose-6,6'-dimycolate, which is more related structurally to cord factor, elicited a stronger response in the BCG-sensitized mice than trehalose-6,6'-dipalmitate. This type of hypersensitivity is distinct from delayed hypersensitivity to tuberculoprotein. In repeated experiments, PPD injected intravenously into mice sensitized with BCG did not induce any observable change in the granulomatous response of mice.

Granuloma formation may represent a defense mechanism where large numbers of macrophages are mobilized at host sites invaded by bacteria that are not disposed of readily. During the infection, the host develops the ability to react with an "accelerated tubercle formation" to combat a reinfection with the same parasite. The effector cells in such a tubercle are main macrophages, which are becoming activated during the process of granuloma formation (6,

10, 15). The process of activation may take place through the intermediary of sensitized lymphocytes or by the direct action of the granulomagenic substance, or both. Cord factor is able to activate macrophages in a direct way (E. Yarkoni et al., manuscript in preparation).

The kind of hypersensitivity induced by the glycolipid component of the BCG cell wall-cord factor differs from that induced by another component, namely tuberculoprotein or PPD. In the latter case, hypersensitivity of the delayed type is induced. In both cases, the substrate on which the materials are presented to the host are important. Neither cord factor in emulsion nor PPD in saline when injected alone are able to induce granulomatous hypersensitivity or hypersensitivity of the delayed type, respectively.

Our findings are in agreement with those of Kawata et al. (12) as well as with the view expressed by Epstein, that tubercle formation in tuberculosis depends on a unique type of hypersensitivity distinct from classic delayed sensitivity (7). During the preparation of this manuscript, a similar paper was published on this subject, using rabbits instead of mice (13). The difference in the species of the animal makes comparison of results difficult because the reaction to administration of cord factor into mice is different from that in the guinea pig and, apparently, in the rabbit. An emulsion of cord factor injected intravenously into guinea pigs does not induce a granulomatous response in their lungs. It is also our experience that cord factor injected into rabbits causes a minimal granulomatous response, different in its cellular composition from that in mice. Since living BCG bacilli do cause a granulomatous response in guinea pigs and rabbits, it is conceivable that in these species the substrate to which cord factor and other granulomagenic substances (like Wax D, all of them components of the mycobacterial cell wall) are bound is important in induction of the granuloma. However, one cannot exclude that the differences in the results obtained in mice, guinea pigs, and rabbits might be due to differences in the purity of cord factors prepared in different laboratories and used in different animal models. These questions are under investigation.

The intensity of the reaction to cord factor used in this laboratory varied even in different strains of mice, as has been mentioned in this work as well as in a previous one (2).

Our findings in the present work raise several questions regarding the antigenicity of cord factor, its sensitizing and granulomagenic activity when given to the host on an appropriate substrate, the role of lymphocytes in granuloma induction, the relationship between granulomatous hypersensitivity and delayed hypersensitivity to protein, and the connection of cord factor with activation of macrophages which form the tubercle. These questions, which are under investigation in this laboratory, are important because the granulomagenic activity of cord factor and other granulomagenic agents may be used in increasing antibody formation to unrelated antigens (4) and in resistance to tumors (5) as well as adjuncts in chemotherapy of tumors. Cord factor and other granulomagenic substances strongly inhibit the growth of Ehrlich ascites cells. (E. Yarkoni et al., manuscript in preparation). This activity is apparently connected with the host cellular reaction induced by these agents (5). Our view is supported by recently published papers in which the connection between the granulomatous response elicited by living BCG bacilli and antitumor activity is strongly emphasized (8, 9).

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