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Immunosuppression induced by talc granulomatosis in the rat

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SUMMARY

Granulomatosis caused by four subcutaneous talc powder-suspension injections induced strong immunosuppression in rats. The disturbance included reduction of mononuclear white blood cell count in the peripheral blood, atrophy of the thymic cortex, spleen enlargement with predominance of red over the white pulp, increase in the number of lymph node germinal centres and a significant delay of the first-set and second-set allograft rejection. Neither phagocytic function of reticulo-endothelial system nor erythrocyte count and humoral immune response were found to be altered. Indomethacin suppression of prostaglandin production did not normalize the allograft rejection dynamics. In contrast, splenectomy completely abolished the immunosuppressive effects of granulomatosis. In splenectomized, talc-treated animals WBC counts were not altered and the rejection of allografts was not delayed. Suppression of immune response to alloantigens was transferred to normal and splenectomized recipients by both serum and spleen cells of talc-injected animals. Also, in a cell mixture-transfer experiment, spleen cells from talc-granulomatosis-bearing donors suppressed the immune response induced by lymph node cells from immune donors in T cell-deficient rats. The inability of serum from splenectomized talc-injected rats to transfer the suppression suggested the crucial role of the spleen in the mechanisms leading to suppression in rats bearing talc-granulomatosis.

Keywords talc immunosuppression splenectomy granuloma

INTRODUCTION

A variety of particles that are poorly degraded by phagocytic cells induce the formation of granulomas *in vivo* (Boros, 1976). The effects of methyl-cellulose (Palmer *et al.*, 1952), silica (Shelley & Hurley, 1960), asbestos (Shorlemmer *et al.*, 1977), bentonite (Boros & Warren, 1973), talc (Eisenman *et al.*, 1947), carageenan (Bonney *et al.*, 1978) and agarose beads (Kobayashi *et al.*, 1985a) have been described. Among these substances, talc has a particular clinical significance inasmuch as its effects in humans were reported after an accidental intake by inhalation (Abraham & Brambilla, 1980), contact with powdered surgical gloves (Sheikh *et al.*, 1984), intravenous injection in heroin addicts (Crouch & Churg, 1983) and injection of crushed tablets (Farber *et al.*, 1982).

A cytotoxic effect of talc on macrophages as well as talc-induced inhibition of lymphocyte proliferation *in vitro* have been well documented (Davies *et al.*, 1983; Hoffelt, 1983), but much less is known about the mechanisms of talc-induced changes *in vivo*. Intravenous injection of talc causes primary endothelial injury leading to thrombosis, perivascular granulomas and massive fibrosis of the lungs (Crouch & Churg, 1983) Talc-induced granuloma can cause severe ovary damage (Hamilton *et al.*, 1984) and significant bone loss (Vukičević *et al.*, 1987).

Our present study was aimed at a detailed dissection of immunological effects of talc granulomatosis in rats. Significant immunosuppressive effects of talc granulomatosis were demonstrated and some of their mechanisms elucidated.

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MATERIALS AND METHODS

Inbred rats of WVM strain (derived from the Wistar stock) and both sexes were used at the age of 3–5 months. Rats of inbred Fisher strain were used as donors of allogeneic skin grafts. T cell-deficient rats (ATXBM) were prepared as described earlier (Vidović *et al.*, 1982). Cell suspension and transfer, preparation of sera and anti-mouse RBC haemagglutinin titre determination and tail-to-tail skin grafting have also been described in detail (Vidović *et al.*, 1982).

Talc, $Mg_3H_2(SiO_3)_4$, was a commercial powder preparation (Jugohospitalija, Zagreb) commonly used for hospital purposes. It was sterilized by heating at 160°C for 1 h, cooled and suspended in phosphate-buffered saline (PBS), 1 g/ml. It was injected at four subcutaneous injection sites on the animals backs, to a total of 1 g/rat. The dose was chosen according to our

Table 1. Time course of the changes in peripheral white blood cell (WBC) counts and in the spleen of rats with talc-induced granulomas

Days after talc injection	WBC ($\times 10^9$/l)			Spleen	
	Total	Mononuclears	Polymorphonuclears	Weight (g/kg BW)	Number of cells ($\times 10^6$)
Control	13·3±2·1	11·4±1·2	2·0±0·8	2·4±0·3	232·9±17·2
3	10·3±2·4 (NS)	8·4±1·5 (NS)	1·9±0·6 (NS)	3·0±0·7 (NS)	ND
5	5·5±1·2 ($P<0·01$)	3·7±1·3 ($P<0·01$)	1·8±1·1 (NS)	2·9±0·2 (NS)	ND
7	6·3±0·2 ($P<0·01$)	3·6±1·5 ($P<0·01$)	2·7±0·9 (NS)	3·8±0·6 ($P<0·05$)	451·6±21·2 ($P<0·01$)
14	15·2±1·4 (NS)	11·8±1·9 (NS)	2·8±0·8 (NS)	5·0±1·4 ($P<0·05$)	720·8±33·5 ($P<0·01$)
21	13·4±1·8 (NS)	10·8±1·7 (NS)	3·7±1·5 ($P<0·05$)	3·7±0·6 ($P<0·05$)	421·6± 7·2 ($P<0·01$)

The values are expressed as arithmetical mean ± s.e.m. The level of significance in comparison to the control is given in parentheses. Six to nine rats per group.

NS, not significant.

ND, not done.

previous experience of its significant effect on bone loss (unpublished).

Splenectomy was always performed 14 days before talc injection, using ether narcosis and electrocauterization of splenic blood vessels and ligaments.

Differential cell blood count at 200 cells/rat was determined after peripheral blood had been smeared on a glass slide, air-dried and fixed and stained with May-Grünwald-Giemsa stain.

Histology and morphometry were performed in rats killed by cervical dislocation 15 h, 3, 7, or 14 days after the talc injection. Spleens, thymuses and axillary lymph nodes were fixed in Bouin's solution, dehydrated and embedded in Paraplast (Lancer, Ireland). Serial 7 μm thick sections were stained with hemalaum-eosine. A fraction of the lymphoid organ area occupied by a distinctive compartment ($\times 25$) was determined using a semi-automatic image analyser (Morphomat 10, Opton, FRG), on at least five serial sections. These were 140, 210 and 280 μm apart for the lymph nodes, spleen and thymus respectively. Thymuses and lymph nodes were cut tangentially, whereas the pieces from the middle part of the spleens were cut orthogonally to their longer diameter. Cellularity of the lymphoid organs was determined by counting the intersections of a Zeiss II ocular grid over the cells ($\times 400$). The mean number of germinal centres per section area of the lymph nodes was determined by counting germinal centres in at least six serial sections.

Indomethacin (Belupo, Zagreb) was dissolved in 1·4% $NaHCO_3$ solution and injected i.p., 2 mg/kg body weight.

Clearance of carbon particles from the blood was used to test macrophage function in the treated rats (Benacerraf *et al.*, 1957). Briefly, carbon particles (C/11/1431a, Günter-Wagner Hannover, FRG) were suspended in 2% gelatin (in PBS) at a final concentration of 0·8%, pH 7·2. The suspension was injected in the jugular vein at a dose of 0·16 g/kg body weight. Blood samples (0·1 ml) were obtained from the tail vein after 3 and 15 min and lysed in 3·3 ml Na_2CO_3 solution. Their optical density was determined at 650 nm using a spectrophotometer (Unicam). The phagocytic index (K) was calculated from the formula:

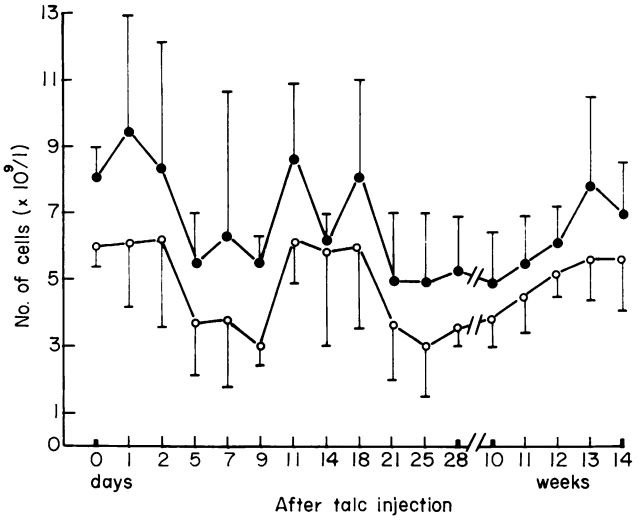

Fig. 1. Absolute numbers of white blood cells (●) and mononuclear cells (○) in the peripheral blood of rats with talc granulomatosis.

$$K=\frac{\log OD_{3\,min}-\log OD_{15\,min}}{12}$$

Corrected phagocytic index α corrects K for the variations of body (BW), liver (L) and spleen (S) weights:

$$\alpha=\frac{BW}{L+S}\times\sqrt[3]{K}.$$

The weights of liver and spleen were determined 2 h after injection of carbon particles (Benacerraf *et al.*, 1957).

Student's t-test was used for statistical analysis throughout the study.

RESULTS

First, we followed white blood cell (WBC) count, and spleen weight and cellularity for 3 weeks after talc injection (Table 1). A significant decrease in WBC count was observed as early as 3

Table 2. Histomorphometric analysis of lymphoid organ changes after subcutaneous talc injection

Lymphoid organ (dimension)	Control ($n=10$)	Finding at the time after talc injection ($\bar{x}\pm$s.d.) ($n=5$)			
		15 h	3 days	7 days	14 days
Thymus					
cortex area (% total)	73·0±3·8	74·2±1·7	55·8±3·5*	77·4±1·0†	74·0±2·3
cellularity (No. × 10^{-3}/mm^2)	40·7±1·5	39·9±1·9	37·2±1·9*	39·5±0·7	40·1±1·9
medulla area (% total)	27·0±3·8	25·8±1·7	44·2±3·5*	22·6±1·0†	25·9±2·3
cellularity (No. × 10^{-3}/mm^2)	22·7±1·8	21·5±0·7	22·5±3·3	22·6±1·0	22·6±2·1
Lymph node (l.n.)					
germinal centres (g.c.)					
area (% total l.n.)	10·2±2·1	16·3±2·8*	18·1±4·5*	6·1±2·3	12·4±1·9
mean g.c. area (mm^2)	0·042±0·006	0·056±0·011	0·053±0·016	0·056±0·008‡	0·094±0·054
No. per l.n. section	8·0±2·0	11·9±1·2‡	13·7±4·3‡	6·8±3·0	9·3±4·4
cellularity (No. × 10^{-3}/mm^2)	37·6±2·5	33·0±1·7†	36·5±1·9	33·0±2·6‡	28·7±2·6*
non-germinal centre cellularity (No. × 10^{-3}/mm^2)	14·7±1·3	17·2±3·7	14·2±2·1	16·0±1·7	15·9±3·1
Spleen					
white pulp area (% total)	10·2±2·1	7·0±0·9†	7·1±1·2†	10·3±1·4	5·9±0·8*
cellularity (No. × 10^{-3}/mm^2)	23·1±1·9	22·8±3·2	26·1±1·5	23·5±0·9	26·7±1·1‡
red pulp area (% total)	63·9±11·6	76·2±1·8‡	77·8±3·6†	64·1±5·0	72·5±2·7
cellularity (No. × 10^{-3}/mm^2)	6·2±0·9	5·2±1·9	6·3±1·8	6·4±1·1	6·8±0·5
macrophage sheet area (% total)	25·9±9·9	17·4±2·1	15·1±3·2*	25·7±3·7	21·6±2·3

*$P<0·001$.
†$P<0·01$.
‡$P<0·05$.

Table 3. Peripheral white blood cell counts and allogeneic skin graft survival in normal and splenectomized rats with talc-induced granuomatosis

Treatment of the recipient rats		White blood cells ($\times 10^9$/l, $\bar{x}\pm$s.e.m.)*			Graft survival time* (days, $\bar{x}\pm$s.e.m.)
Splenectomy	Talc	Total	Mononuclears	Polymorphonuclears	
−	−	15·7±1·3	13·7±1·1	2·0±0·5	16·7±1·4
−	+	7·8±1·2 ($P<0·05$)	6·0±1·7 ($P<0·05$)	1·8±0·9 (NS)	26·3±1·5 ($P<0·01$)
+	−	18·5±2·1 (P<0·05)	15·7±1·8 (NS)	2·8±0·7 (NS)	15·5±1·8 (NS)
+	+	15·3±1·4 (NS)	13·3±1·2 (NS)	2·0±0·6 (NS)	15·0±2·1 (NS)

Rats (8–10 per group) were splenectomized 14 days and treated with talc 7 days before blood analysis and skin grafting, respectively. Blood cell analysis and skin graft rejection experiments were done on different animals.

* Levels of significance in comparison to the control group (first line) are given in parentheses. NS, not significant.

days after talc injection. This was mainly due to a decrease in mononuclear cell number, as the number of polymorphonuclear cells generally remained unchanged (Table 1, three left columns). Two weeks after talc injection, WBC count normalized in relation to both total and mononuclear cell number. Spleen weight and cellularity increased significantly 1 week after talc injection and remained increased for the next 2 weeks (Table 1).

A long-term follow-up of peripheral WBC count (Fig. 1) revealed an early decrease in WBC count and its subsequent recovery, similar to that presented in Table 1. However, a long-term study disclosed another decrease in WBC count which commenced at the end of the third post-injection week and lasted to the end of the third post-injection month (Fig. 1). From day 2 to day 5 after talc injection, peripheral blood erythrocyte number increased by approximately 20%. This difference was not significant (data not shown).

A histomorphometric study of the spleen, lymph nodes and thymus of talc-injected rats (Table 2) revealed a relative decrease in the thymic cortex area and an increase in its medullary area by the third post-injection day, and a reversal of the findings by day 7 after talc injection. A major finding in axillary lymph nodes was an increase in the number of germinal centres within the first

Table 4. Influence of serum and spleen cells from rats with talc granulomatosis on the dynamics of rejection of allogeneic skin grafts; effect of previous splenectomy of donors and/or recipients*

	Donor rats			Skin graft survival in recipients (days, $\bar{x}\pm$s.e.m.)	
Group	Talc	Splenectomy	Transferred material	Non-splenectomized	Splenectomized
1	−	−	—	15·7±1·0	18·7±1·6
2	−	−	serum	16·6±1·1	ND
3	−	−	spleen cells	18·2±3·6	ND
4	+	−	serum	27·2±1·6†	33·8±1·2†
5	+	−	spleen cells	30·4±2·1†	26·8±3·2†
6	+	+	serum	16·3±0·6	ND

* Splenectomy was performed 14 days before talc injection (donors) or skin grafting (recipients). Serum and cell donor rats were injected with talc 7 days before they were killed. Serum and cell recipients were grafted with allogeneic skin immediately after injection of 1·0 ml of serum or 75×10^6 spleen cells i.v.

† Significantly different (at $P<0{\cdot}01$ level) from group 1.

ND, not done.

Table 5. Survival of allogeneic skin grafts in T cell deficient (ATXBM*) rats reconstituted with lymph node cells from syngeneic alloimmune donors and spleen cells from rats with talc-granulomas†

	Treatment of recipient TIR rats		
Group	Lymph node cells	Spleen cells	Graft survival time‡ (days, $\bar{x}\pm$s.e.m.)
1	−	−	76·4±5·0
2	+	−	26·0±3·6
3	+	normal	26·9±2·1
4	+	from talc-treated rats	54·5±6·8

* Thymectomized, lethally irradiated rats reconstituted with syngeneic bone marrow cells. In this experiment, they were used 2 months after irradiation and bone marrow reconstitution.

† On the day of skin grafting, recipient ATXBM rats (9–11 per group) were injected i.v. with 50×10^6 lymph node cells from the donors previously grafted three times with the allogeneic skin grafts and 150×10^6 spleen cells from either normal rats or from rats injected with talc 7 days earlier.

‡ Statistics (*t*-test, $P<$): Groups 1 and 2, 0·001; 1 and 3, 0·001; 1 and 4, 0·01; 2 and 3, NS; 2 and 4, 0·01; 3 and 4, 0·01.

3 days after talc injection. During this period splenic white pulp and macrophage sheet area shrank whilst red pulp area enlarged (Table 2).

The effect of granulomatosis on immune response was tested using haemagglutinin production and allogeneic skin grafting. The analysis of WBC counts in talc-treated rats (Table 1, Fig. 1) prompted us to choose the seventh post-injection day for allograft transplantation.

Haemagglutinin titres in rats injected with mouse red blood cells ranged from 1/4 to 1/64, which was not significantly different from those obtained in rats that were not injected with talc (data not shown). In contrast, a profound inhibitory effect of talc-induced granulomatosis on both first-set and second-set allogeneic skin graft rejection was observed. In talc-injected rats the first allograft was rejected within 27·7±1·45 days (16·6±0·21 days in controls), and the second one, placed 120 days after the first one, was rejected within 20·0±1·8 days (14·0±1·24 days in controls), both delays being significant at the level of $P<0{\cdot}01$. However, the prolongation of allogeneic graft survival by talc-granulomatosis was abolished in splenectomized recipients (Table 3, right column). Splenectomy also protected the talc-injected rats from disturbances in WBC counts (Table 3, three left columns). Talc granulomatosis significantly decreased WBC counts in normal recipients but did not influence their numbers in splenectomized rats.

The data in Table 4 indicate that both serum and spleen cells from talc-injected rats (taken 7 days after talc injection) suppressed allogeneic skin graft rejection in syngeneic recipients, regardless of whether or not the recipients had been splenectomized. However, when the serum from splectomized talc-injected rats was transferred to normal syngeneic recipients, it exerted no influence on the survival of allogeneic skin grafts, but in the same experiment the serum from non-splenectomized talc-injected donors conferred a strong suppression to the recipients (Table 4).

The ability of spleen cells from talc-treated rats to suppress the immune response was confirmed in a cell mixture-transfer experiment, where T cell-deficient ATXBM rats were used as recipients (Table 5). ATXBM rats rejected allogeneic skin grafts in 76 days, but a single injection of lymph node cells from alloimune donors accelerated the rejection to 26 days. However, when the spleen cells from talc-injected rats were admixed to the lymph node cells from alloimmune donors, the rejection of allografts in TIR recipients was shown to be significantly prolonged again.

The next two experiments were performed to test the possible prostaglandin and/or macrophage function alterations in talc-injected rats. To investigate whether the prostaglandins

released from the granuloma-forming cells induced a splenic suppressor activity, indomethacin, an inhibitor of prostaglandin synthesis (Pope, 1985), was injected i.p. to talc-treated rats, in a dose of 2 mg/kg body weight daily for 12 days, starting on the day of talc injection. Allogeneic skin was grafted 7 days after talc injection. Indomethacin treatment completely failed to reverse the delay of allograft injection as achieved in talc-treated recipients. Thus, the allograft median survival time did not differ between the talc-treated and the talc-plus-indomethacin-treated rats (data not shown).

The macrophage function was tested by measuring the rate of clearance of carbon particles from the blood in normal and talc-treated rats. Carbon clearance was determined 3, 5 and 10 days after talc injection. In both normal and talc-treated rats, the phagocytic index K ranged 0·08–0·15 and the corrected phagocytic index α was 10·5–15·5, revealing no difference in macrophage function between the two groups studied (data not shown).

DISCUSSION

Studies with neo-natally thymectomized rats, rats injected with anti-thymocyte serum (Nago *et al.*, 1981) and nude rats devoid of functional T cells (Tanaka *et al.*, 1982) have demonstrated that granuloma formation *in vivo* (the so-called 'foreign body' granuloma) does not require T cell-dependent immunological reactions. On the other hand, granuloma formation profoundly impairs the immune response. It appears, however, that the changes affected predominantly the cellular immune response. For example, in mice with murine leprosy cellular immune response was severely diminished, whereas humoral response remained undisturbed (Ptak *et al.*, 1970). In this study, talc-induced granuloma formation affected both primary and secondary cellular immune response but had no influence on either humoral immune response or phagocytic function of the reticuloendothelial system (data not shown). Mononuclear cells disappeared temporarily from the peripheral blood and the spleen was enlarged (Table 1). Thymic cortex atrophy was not surprising (Table 2). However, the early increase in the number of lymph node germinal centres and the shrinking of splenic white pulp (Table 2) should be related to specific pathogenesis of talc granulomatosis and further studied.

Similar changes in peripheral blood and spleen in parallel with immunosuppression have been ascribed to the atrophy of lymphoid system, or to disturbance of lymphocyte circulation in granulomatous disorders (Bullock *et al.*, 1976). The increased cellularity of the spleen in talc-treated rats could be due to either increased local production or to accumulation ('trapping') of cells (Bullock *et al.*, 1976).

Our study also revealed that the decrease in cellular immune response was not a consequence of the lack of immunocompetent cells, but of an active suppression, of a definite duration, in which the spleens of the affected animals played an important role. Thus, the transfer of spleen cells or serum from talc-treated rats delayed allogeneic skin graft rejection in normal rats, and the splenectomy of the talc recipients completely abolished the immunosuppressive effects of talc granulomatosis (Table 3).

These findings correlated with the normalization of WBC count in talc-treated rats which had been splenectomized before talc injection (Table 3). This observation corresponds to those of Old *et al.* (1962) and Bullock *et al.* (1976), which showed the beneficial effects of splenectomy on rat immune response and WBC count, and to the work of Kobayashi *et al.* (1985a) which demonstrated the suppressor cells in granuloma-bearing animals. These findings also support the observation that the spleen acts to generate suppressor cells during the contact-sensitivity reactions (Sy *et al.*, 1977).

On the other hand, both serum and spleen cells from talc-treated donors were able to transfer suppression to both splenectomized and non-splenectomized recipients (Table 4). This further confirmed the suppressive abilities of the spleen cells, but revealed a serum effect from the talc-treated rats. However, since the serum of splenectomized talc-treated donors was not suppressive (Table 4), we must conclude that the suppressive factors in the serum were of splenic and not of pre-splenic origin. Nevertheless, the relationship between these immunosuppresive humoral factors from the spleen and the immunosuppressive splenic cells (Tables 4 and 5) is not clear. It is possible that the spleen produced both suppressive elements, cellular first and then humoral, or that the spleen produced a humoral factor which rendered the potentially suppressive cells functional.

The relationship between the granuloma and the spleen in the talc-treated rats remains obscure. Since the serum from splenectomized talc-treated rats was not suppressive (Table 4), it is obvious that the granuloma itself did not secrete such factors. This is in accord with the findings of Kobayashi *et al.* (1985a), who could not detect suppressor activity in the granuloma extract from granuloma-bearing mice, although the mice were clearly immunosuppressed and possessed suppressor cells in their lymph nodes. On the other hand, one should conjecture that the granuloma may have triggered the series of events which finally conferred suppression to the treated recipients (Allred *et al.*, 1985). We tested the possibility that prostaglandin production might precipitate the suppression chain of events, as has been reported by others in different experimental systems (Goodwin *et al.*, 1977; Pope, 1985). However, even relatively high doses of indomethacin (2 mg/kg/day) were completely ineffective, although the treatment started simultaneously with talc injection and lasted until 4 days after skin grafting (performed 7 days after talc injection).

The resistance of humoral immune response and phagocytic activity of the reticuloendothelial system to the suppressive effects remains unexplained, as does the relationship between the granuloma and the spleen. We believe that hormonal and lymphokine changes may underlie the overall changes which have been observed: for example, MIF and IL-1 activities in the granuloma tissue, as well as an impaired IL-2 production in granuloma-bearing mice, have been documented previously (Kobayashi *et al.*, 1985; 1985a). However, the fact that an immunostimulatory protein has also been isolated from mouse talc-induced granuloma (Fontan *et al.*, 1983) further illustrates the complexity and multitude of the processes and events which occur in the course of granuloma formation.

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