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Induction of cytotoxic peritoneal exudate cells by T-cell immune adjuvants of the β(1→3) glucan-type lentinan and its analogues

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Summary. Eight distinct polysaccharides (PS) of β(1→3) glucan type were tested for their capacity to render murine peritoneal exudate cells (PEC) cytotoxic. After intraperitoneal injection of lentinan, pachymaran and HE-pachyman 3 and 4 highly cytotoxic PEC were induced. Pachyman and HE-pachyman 1 and 2 were of moderate effect, whereas CM-pachymaran and HE-pachyman 3 and 4, highly cytotoxic PEC were induced. Pachyman and HE-pachy-macrophages. The induction of PEC-dependent cytotoxicity exhibited a strict dose relationship. Optimal administration of PS resulted in the induction of cytotoxicity, which persisted for more than 25 days. Surprisingly, none of the PS tested was capable of rendering normal or thioglycollate-induced PEC cytotoxic under *in vitro* conditions. It is suggested that the capacity of PS to render *in vivo* macrophages cytotoxic is related to the potency of these PS to activate the alternative pathway of complement system (APC) in so far as C3b may be the essential component required to render macrophages cytotoxic.

Abbreviations: APC, alternative pathway of complement activation; BCG, Bacillus Calmette-Guérin; FCA, Freund's complete adjuvant; CM, carboxymethyl; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FCS, foetal calf serum; HE, hydroxyethym; LPS, lipopolysaccharide; MLC, mixed lymphocytes culture; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PS, polysaccharides; S 180, sarcoma 180.

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INTRODUCTION

Because of its theoretical and possibly practical implications, tumour immunotherapy has attracted much interest. For example, immune adjuvants such as BCG (Biozzi, Stiffel, Halpern & Mouton, 1959; Old, Clarker & Benacerraf, 1959; Germain, Williams & Benacerraf, 1975; Pimm & Baldwin, 1975), anaerobic coryne forms (Halpern, Biozzi, Stiffel & Mouton, 1966; Castro, 1974) or β(1→3) glucans (Chihara,Maeda, Hamuro, Sasaki & Fukuoka, 1969; Chihara, Hamuro, Maeda, Arai & Fukuoka, 1979a; Hamuro, Yamashita, Ohsaka, Maeda & Chihara, 1971), have been shown in animal models to enhance protective immunity to transplanted tumours. Probably because of their remarkably complex chemical nature, the active entities of the former two have not yet been fully characterized, and their mode of action is still poorly understood. In contrast, the latter substances have been well characterized (Chihara, Hamuro, Maeda, Arai & Fukuoka, 1970b; Hamuro, Maeda, Chihara & Fukuoka, 1976) and their *in vivo* applications appear not to be associated with toxic side effects (Maeda, Hamuro, Yamada, Ishimura & Chihara, 1973). An analysis of their mode of action, therefore, may pro-

vide a rationale for their controlled use to enhance protective immunity in tumour-bearing hosts.

There is circumstantial evidence that the β(1→3) glucan, lentinan, preferentially acts on T cells (Allison, 1973; Dresser & Phillips, 1974), and possibly enhances T-helper cell functions (Dennert & Tucker, 1973; Haba, Hamaoka, Takatsu & Kitagawa, 1976). As with other β(1→3) glucans like pachymaran, carboxymethylpachymaran and hydroxyethylpachyman, there is no available information with regard to their immunological reactivities. We, therefore, re-investigated the effect of β(1→3) glucans on various parameters of cell-mediated immunity. Until now we have observed that the β(1→3) glucans lentinan, pachyman, pachymaran and HE-pachymans strongly enhanced the capacity of mice to generate alloreactive cytotoxic T lymphocytes (CTL) in response to allogenic tumours (Hamuro, Röllinghoff & Wagner, 1978c). In addition when added to mixed lymphocyte culture (MLC), β(1→3) glucans enhanced the lytic activity of the alloreactive CTL generated (Hamuro, Wagner & Röllinghoff, 1978b).

In the course of these studies it was noted that β(1→3) glucans not only enhanced via an indirect mechanism the responsiveness of antigenically triggered CTL precursors, but also affected the reactivity of peritoneal exudate cells (PEC). Many studies indicate that macrophages activated by specific or non-specific means also contribute to the suppression of tumour growth (Alexander & Evans, 1971; Hibbs, Lambert & Remington, 1972; Keller, 1973; Nathan, Hill & Terry, 1976; Russell & McIntosh, 1977), in addition to the immunological roles displayed by lymphocytes (Evans & Alexander, 1972a, b; Pfizenmaier, Trostmann, Röllinghoff & Wagner, 1975; Piessens, Churchill & David, 1975; North & Kirstein, 1977). Here we describe that after i.p. injection of β(1→3) glucans into mice, the adherent peritoneal cells (macrophages) were cytotoxic *in vitro* against tumour target cells. However, the same polysaccharides (PS) failed to render PEC cytotoxic under *in vitro* conditions. The differential effects of β(1→3) glucans on PEC and on T-cell mediated immunity will be discussed in relation to their mode of action as immune adjuvants.

MATERIALS AND METHODS

Polysaccharides of β(1→3) glucan types

Lentinan was extracted from fruit bodies of Lentinus edodes (Berk.) Sing and purified as described previously (Chihara *et al.*, 1969). Pachyman was extracted from *Poria cocos* Wolf and pachymaran was obtained by chemical modification of pachyman (Chihara *et al.*, 1970a). Carboxymethylpachymaran (CM-pachymaran) was synthesized from pachymaran by carboxylmethylation (Hamuro *et al.*, 1971), the degree of substitution with CM groups was 0·72. Hydroxyethylpachyman (HE-pachyman) was derived from pachyman by hydroxyethylation (Hamuro *et al.*, 1976) and the degrees of substitution with HE groups were respectively 0·09, 0·11, 0·26 and 0·43, for HE-pachyman-1, -2, -3, -4. β(1→3) glucan suspension was made by homogenizing 25 mg of a given β(1→3) glucan with 5 ml of saline in a teflon homogenizer for 5 min at room temperature and sterilizing at 120° for 30 min. The β(1→3) glucans were a generous gift from Ajinomoto Co., Inc., Tokyo, Japan.

Mice

Adult mice of the inbred strains CBA/J, C57Bl/6, BALB/c, A and DBA/2 were purchased from G1. Bomholtgaard, Ry, Denmark.

Target tumour cells

P 815-X mastocytoma cells (H-2^d), EL4 thymoma cells (H-2^b) were grown in continuous suspension cultures. The F9 cells were cultivated in tissue culture dishes with gelatine in Dulbecco's modified Eagle's medium containing 15% foetal calf serum in an atmosphere of 10% CO_2. The PCC3, PCC4 cells lines were cultivated similarly without gelatine (Wagner, Starzinski-Powitz, Röllinghoff, Golstein & Jakob, 1978). The F9, PCC3 and PCC4 were harvested using 1 mM EDTA in phosphate-buffered saline (pH 7·3) free of Mg^{2+} and Ca^{2+}. Con A transformed lymphoblasts were obtained by pooling replicate cultures containing 4×10^6 spleen cells cultured for 72 h in DMEM in the presence of 5 μg/ml Con A. Usually $1–2 \times 10^6$ cells were labelled with 150 μCi ^{51}Cr (Radiochemical Centre, Amersham) as described previously (Wagner, Goetze, Ptschelinzew & Röllinghoff, 1975).

Injection of β(1→3) glucans

At a given dose of PS, the following volumes of sterile β(1→3) glucan suspension were inoculated using different routes: i.p. 0·5 ml; i.m. 0·1 ml; s.c. 0·1 ml; i.d. 0·05 ml.

Peritoneal exudate cells

At an appropriate interval after PS inoculation, 3 ml of

PEC were harvested by lavaging with 5 ml sterile saline containing 10 U heparin/ml. After washing twice with saline, the cells were resuspended in 3 ml DMEM. In the direct assay of cytotoxicity a constant volume of this cell suspension was incubated with the target cells. To fractionate the adherent from non-adherent cells, the cell suspension was incubated in Linbro plates for 3 h at 37° and the non-adherent cells were removed by extensive washing with DMEM.

Cytotoxic assay

The cytotoxic activity of induced PEC was tested in several ways. First, a constant volume of PEC suspension was diluted with DMEM and 1 ml of the diluted cell suspension was then incubated in the presence of 100 μl of ^{51}Cr-labelled target cells for 18 h at 37° in 7·5% CO_2 atmosphere in round-bottomed plastic tubes (Greiner, Nürtingen, W.-Germany, lot No. 175). Second, the PEC harvested were adjusted to contain an equal number of cells induced and tested for cytotoxicity for 18 h. Third, the PEC were fractionated into adherent and non-adherent cell populations by incubating them for 3 h in Linbro plates. Thereafter, the adherent PEC were collected, counted and transferred into tubes. 2×10^4 ^{51}Cr-labelled target cells in 100 μl DMEM were then added and after an 18 h incubation the extent of cytotoxicity was assayed. The radioactivity of both supernatant and pellet was determined in a Packard autogamma counter. Percentage lysis was calculated according to the formula:

$$\% \text{ lysis} = \frac{^{51}\text{Cr-release by PEC} - ^{51}\text{Cr- release of control}}{\text{maximum } ^{51}\text{Cr-release} - ^{51}\text{Cr-release of control}} \times 100$$

RESULTS

The number of peritoneal exudate cells (PEC) obtained from mice 3 days after i.p. injection of an increasing dose of the β(1→3) glucan pachymaran was found to be strongly increased in a dose-dependent manner. Thus a six-fold increase in the number of PEC was obtained after i.p. injection of 50 mg/kg (three times) when a constant volume of wash-out fluid was used to harvest the PEC (Table 1). Unlike the PEC obtained from control mice, the PEC from pachymaran-treated mice at various cell concentrations exhibited strong cytotoxic activity towards ^{51}Cr-labelled P 815 (H-2^d) target cells. The magnitude of lysis observed in the 18 h cytotoxicity test was further increased provided the PEC were pre-incubated for 24 h at 37° prior to ^{51}Cr-assay (Table 1). From the data available it can be concluded that the lytic activity of the PEC tested did not correlate with the overall number of PEC induced. Thus on a cell to cell basis, PEC from mice treated three times with 1 mg/kg pachymaran exhibited higher cytotoxic activity than

Table 1. Induction of cytotoxic peritoneal exudate cells (PEC) by pachymaran

Daily dose of pachymaran (mg/kg) at three consecutive days*	Number of PEC ($\times 10^{-4}$)/ml	% Lysis of ^{51}Cr-labelled P 815†							
		Direct assay				Assay after preincubation of the PEC for 24 h			
		1:1‡	1:3	1:9	1:27	1:1	1:3	1:9	1:27
1	212	47	37	30	25	70	50	28	4
10	274	48	31	31	17	81	56	25	2
50	464	71	35	36	20	65	83	62	1
Control	82	4	2	ND§	ND	0	0	ND	ND

* CBA/J mice were injected i.p. with increasing doses of pachymaran on three consecutive days. One day later a fixed amount, 3 ml, of peritoneal wash out fluid was collected and the cells obtained were counted.

† The cytotoxic activity of the PEC was tested in a 18 h cytotoxicity assay against 2×10^4 P 815 target cells. The assay was performed either immediately after the collection of the PEC or after a further cultivation period of PEC for 24 h at 37°. Background lysis was less than 32%.

‡ Dilution of the peritoneal wash out fluid.

§ ND, not done.

Table 2. Cytotoxicity of pachymaran-activated PEC at different time intervals after pachymaran administration

Daily dose of pachymaran (mg/kg) on three consecutive days*	% Lysis of ^{51}Cr-labelled P 815 cells			
	Day 3 PEC† (directly)	Day 3 PEC (after 24 h cultivation)	Day 10 PEC (directly)	Day 10 PEC (after 24 h cultivation)
1	41	73	40	95
10	38	80	43	100
50	29	75	0	0
Control	4	0	6	−1

* Pachymaran was administered as described in Table 1.

† PEC were collected either at day 3 or day 10 after the first i.p. injection with pachymaran. The 18 h ^{51}Cr-release assay was performed similarly as described in Table 1. The ratio of unfractionated PEC to target cells was 20:1. Background lysis was less than 32%.

those from mice treated with higher doses of pachymaran.

The results given in Table 2 were obtained at a ratio of effector to target cells of 20:1 and indicate that the cytotoxic activity of PEC obtained from mice treated three times with 50 mg/kg pachymaran was negative 10 days after injection. In contrast, at this time point PEC from mice treated with lower doses of pachymaran still exhibited highly lytic activity. In screening various $\beta(1\rightarrow3)$ glucans for their capacity to induce *in vivo* cytotoxic PEC, a constant dose of 10 mg/kg of lentinan, pachyman, pachymaran, CM-pachymaran and four distinct HE-pachymans (-1, -2, -3, -4) were administered i.p. to CBA mice on day 0, 1 and 2. The PEC were harvested from the different groups of mice either at day 3 or at day 6 and tested for cytotoxic activity. Lentinan, pachymaran, and HE-pachyman-3 and -4 induced strongly cytotoxic PEC within 3 days, after 6 days the cytotoxicity even appeared to be increased. In contrast, no cytotoxic activity was observed at day 3 with PEC from mice injected with CM-pachymaran, pachyman and HE-pachyman-1.

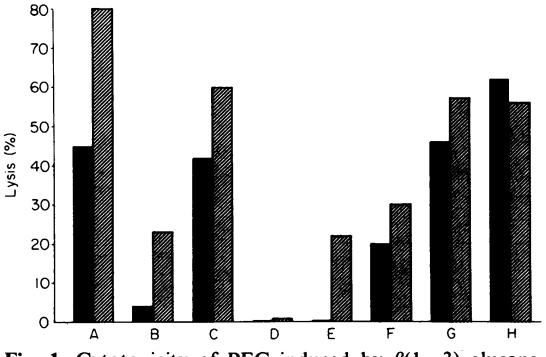

Fig. 1. Cytotoxicity of PEC induced by $\beta(1\rightarrow3)$ glucans. Polysaccharides (10 mg/kg) were injected ip into CBA/J mice on day 0 and PEC were collected on day 3 (filled column) or on day 6 (hatched column). The cytotoxicity of PEC obtained was tested in a 18 h ^{51}Cr-release assay. The ratio of unfractionated PEC to P 815 target cells was 20:1. The lysis of control group (PEC from normal mice) was below 7% and the background lysis was less than 28%. A, Lentinan; B, pachyman; C, pachymaran; D, CM-pachymaran; E, HE-pachyman-1; F, HE-pachyman-2; G, HE-pachyman-3; H, HE-pachyman-4.

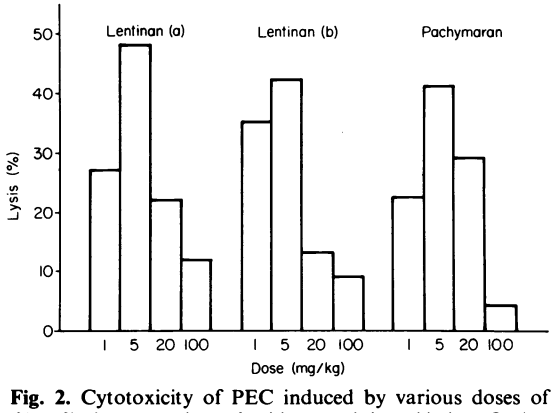

Fig. 2. Cytotoxicity of PEC induced by various doses of $\beta(1\rightarrow3)$ glucans. Polysaccharides were injected ip into CBA/J mice and cytotoxicity of PEC harvested on day 3 was assessed in a 18 h ^{51}Cr release assay. Lentinan (a): injection on day 0; lentinan (b), pachymaran: injection on day 0, 1, 2.
The ratio of unfractionated PEC to P 815 target cells was 20:1. The control lysis was 4·3% and the background lysis was 36%.

Table 3. Effect of injecting β(1→3) glucans i.p. at day 0 and at day 10 on numbers and cytotoxicity of PEC

	Number of PEC ($\times 10^{-4}$/ml)†				% lysis of ^{51}Cr-labelled P 815 cells			
β(1→3) glucan*	Day 3	Day 6	Day 16	Day 25	Day 3	Day 6	Day 16	Day 25
Lentinan	212	188	280	266	40	5	29	64
Pachyman	224	166	150	240	10	0	10	66
Pachymaran	374	178	284	296	22	3	34	34
Control	106	118	168	250	2	0	3	2

* CBA/J mice were injected i.p. with β(1→3) glucans of the indicated type (50 mg/kg) at day 0 and received a second injection (2 mg/kg) at day 10 (i.p.).

† At several time points PEC were collected, counted and tested for cytotoxicity in an 18 h ^{51}Cr-release assay. The ratio of unfractionated PEC to target cells was 20:1. Background lysis was less than 38%.

The latter two, however, induced moderate cytotoxicity when tested after 6 days (Fig. 1). The results depicted in Fig. 2 suggest a strict dose–response relationship between the amount of β(1→3) glucans administered and the magnitude of cytotoxicity induced. Accordingly independent of the injection schedule used, 5 mg/kg of lentinan or pachymaran induced the highest cytotoxic activity.

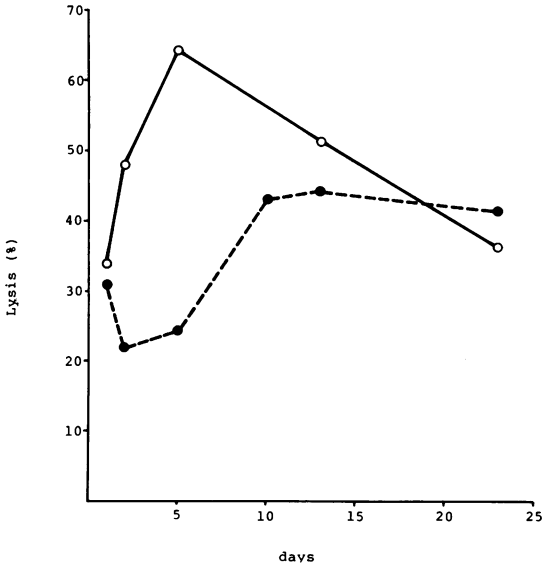

Fig. 3. Duration of cytotoxicity of PEC induced by pachymaran. Pachymaran was injected on day 0, 1, 2 at a dose of 1 mg/kg (○) or of 10 mg/kg (●). 18 h ^{51}Cr-release assays were carried out at a ratio of unfractionated PEC to P 815 target cells of 20:1. Control lysis was below 3%, and background lysis was less than 38%.

In the next set of experiments, it was tested whether cytotoxic PEC could again be induced in mice which had already received high doses of β(1→3) glucans and therefore did not contain cytotoxic PEC after 10 days, by rechallenging on day 10 with lower doses of β(1→3) glucans. As can be seen in Table 3, highly cytotoxic PEC were indeed found at day 16 which persisted until day 25. Again it should be stressed that there was no obvious correlation between the number of PEC induced and the magnitude of cytotoxicity observed. This finding was further substantiated in experiments aimed at an analysis of the kinetics of the influx of PEC and their respective cytotoxic activity in β(1→3) glucan treated mice. The results obtained with two different doses of pachymaran demonstrated that from day 2 until day 23 almost no difference in the number of PEC harvested. In contrast, PEC of mice treated three times with 1 mg/kg of pachymaran exhibited peak cytotoxicity at day 5 which gradually decreased thereafter. PEC from mice treated three times with 10 mg/kg exhibited low cytotoxic activity at day 5 and reached a plateau between days 10 and 25 (Fig. 3).

Due to the ease with which cytotoxic effector cells can be induced in PEC by i.p. injection of β(1→3) glucans the question may arise whether the local inflammation caused by a high local concentration of PS is the reason PEC activation takes place. Surprisingly, s.c. injection of β(1→3) glucans induced as efficient cytotoxic PEC as local i.p. injection; i.m., i.d., and i.v. injections were slightly less effective (Fig. 4). These results further stress the effectiveness of β(1→3) glucans in rendering *in vivo* PEC cytotoxic. In agreement with published work (Hibbs *et al.*, 1972; Cleveland, Metzger & Zbar, 1974; Germain *et al.*, 1975) no

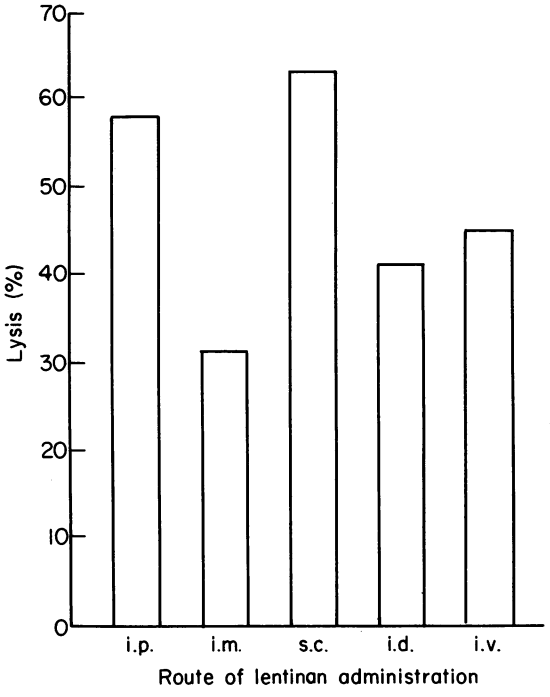

Fig. 4. Cytotoxicity of PEC induced by lentinan after administration via different routes. Lentinan (10 mg/kg) was injected at day 0, 1, 2 and 18 h ^{51}Cr-release cytotoxic assays were performed at day 10 with the ratio of unfractionated PEC to P 815 target cells of 20:1. The intradermal injection was carried out in the food pad. Control lysis was below 5% and background lysis was less than 36%.

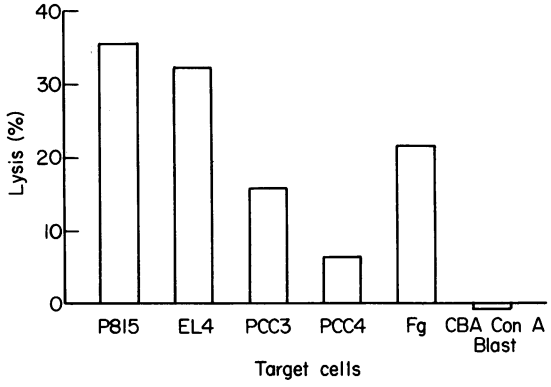

Fig. 5. The effect of pachymaran-induced cytotoxic PEC on different target cells. Pachymaran (10 mg/kg) was injected i.p. into CBA/J mice on day 0, 1, 2 and 18 h ^{51}Cr-release assays were performed at day 0 with the ratio of adherent PEC to target cells of 10:1. Background lysis was less than 52%. The lysis (%) was calculated after correction for the control lysis, which was taken as 0%.

antigen specificity of the $\beta(1\rightarrow3)$ glucan-induced cytotoxic PEC could be observed. When they were tested against an array of target cells, lysis of antigenically unrelated tumour target cells was observed (Fig. 5). Con A-induced syngeneic blast cells, however, were not lysed. It should be noted that $\beta(1\rightarrow3)$ glucans induced equally cytotoxic PEC in CBA, BALB/c, C57Bl/6, and DBA/2 mice (data not given).

Despite many experiments to date, all our attempts to render normal or thioglycollate induced PEC cytotoxic by the *in vitro* addition of $\beta(1\rightarrow3)$ glucans have failed. Using all seven $\beta(1\rightarrow3)$ glucans over a wide range of different concentrations and incubating time in the presence or absence of FCS over various time intervals *in vitro*, no significant cytotoxic activity could be induced. The *in vitro* incubation of normal PEC with culture supernatant of spleen cells, obtained from PS sensitized (i.p.) mice, in the presence or absence of PS also failed to render PEC cytotoxic. On the basis of these negative results (data not shown), we conclude that $\beta(1\rightarrow3)$ glucans are capable of rendering PEC cytotoxic only under *in vivo* conditions.

DISCUSSION

The results presented here suggest that a variety of different $\beta(1\rightarrow3)$ glucans are capable of inducing highly cytotoxic PEC *in vivo*. Of eight different $\beta(1\rightarrow3)$ glucans tested, lentinan, pachymaran, HE-pachyman-3 and 4 were found to be most effective. In contrast, CM-pachymaran failed to induce any activity; pachyman and HE-pachyman-1, 2 had a moderate effect. Each active $\beta(1\rightarrow3)$ glucan exhibited a strict dose–response relationship such that at high doses the cytotoxic activity of PEC was drastically reduced. Although all of the $\beta(1\rightarrow3)$ glucans except for CM-pachymaran dramatically but transiently increased the number of PEC in treated mice, there was no correlation between the numbers of PEC harvested and the magnitude of cytotoxicity observed. The kinetics of induction of cytotoxic PEC was found to be dose-dependent, whereby cytotoxicity persisted for more than 23 days. The cell type responsible within the PEC was an adherent, Ig-negative, θ-negative cell. Since cytotoxicity increased upon *in vitro* incubation at 37°, a procedure which inactivates natural killer cells (Herberman, Nunn & Larsi, 1975; Kiessling, Klein & Wigzell, 1975; Wolfe, Tracey & Henney, 1977) and carbonyl iron treatment completely reduced the

cytotoxicity, it was tentatively concluded that macrophages were mediating the immunologically non-specific lytic activity observed. Experiments are presently being performed to substantiate further the macrophage nature of the cytotoxic effector cells.

To date many substances have been reported to induce *in vivo* cytotoxic or cytostatic macrophages, for example BCG (Cleveland *et al.*, 1974; Germain *et al.*, 1975), *Corynebacterium parvum* (Christie & Bomford, 1975), poly I:C (Alexander *et al.*, 1971), pyran (Kaplan, Morahan & Regelson, 1974), Freund's complete adjuvant (Hibbs *et al.*, 1974), lipopolysaccharide (Alexander *et al.*, 1971) and interferon (Schultz, Chirgos & Heine, 1978). However almost all of them are of complex chemical nature, in particular those of bacterial origin. In contrast, all of the β(1→3) glucans tested here are purified and chemically defined neutral PS, characterized by the presence of consecutive β(1→3) linked glucose units in the main chain.

CM-pachymaran was found not to induce cytotoxic PEC, although its skeletal structure is identical to that of pachymaran. Obviously the biological difference could be due to the CM groups chemically introduced to pachymaran, which in turn greatly enhanced the water solubility of this substance. If phagocytosis of β(1→3) glucans is an essential step in PEC activation, substances which are not very soluble might be more efficiently phagocytosed. Although the experiments presented here indicate that neutral PS sharing β(1→3) glucosidic linkages as a main chain can render PEC cytotoxic under *in vivo* conditions, the mechanism of this activation is as yet unclear. It is also difficult to explain why β(1→3) glucans are ineffective under *in vitro* conditions, while for example lypopolisaccharides are effective. In this context, it may be important that all of the β(1→3) glucans capable of rendering PEC cytotoxic *in vivo* are efficient in activating the alternative pathway of complement (APC) (Hamuro, Hadding & Bitter-Suermann, 1978a). Only CM-pachymaran, devoid of APC-activating potency, failed to render PEC cytotoxic. Since β(1→3) glucans possessing APC-activating potency could generate *in vivo* active complement components such as C3b, and since C3b in turn is capable of rendering macrophages cytotoxic (Schorlemmer, Hadding, Bitter-Suermann & Allison, 1977b), the biological activity of β(1→3) glucans tested here might be explained by their APC-activating effect.

Our interest in β(1→3) glucans is based on the observation that these substances strongly enhance protective immunity to S180 transplanted tumours (Chihara *et al.*, 1969, 1970a, b; Hamuro *et al.*, 1976). Subsequently, we found that all of the β(1→3) glucans tested here can be classified as T-cell immune adjuvants, because they strongly enhance the generation of alloreactive and H-2 restricted hapten-specific CTL, under both *in vivo* and *in vitro* conditions (Hamuro *et al.*, 1978 b, c). In analysing the mechanism of this adjuvant effect, it was noted that on *in vitro* incubation of splenic adherent cells or PEC with a critical concentration of β(1→3) glucans, a soluble product is generated which in turn is able to augment the differentiation of antigenically triggered CTL precursors into highly reactive CTL (unpublished observations). Therefore, under *in vivo* conditions β(1→3) glucans may trigger at least two independent events, the first rendering macrophages cytotoxic and the second enhancing the *in vivo* generation of CTL by triggering the production of 'factors' influencing the differentiation of precursor CTL. If this conclusion is correct, both mechanisms may act synergistically during *in vivo* rejection of transplanted S180 tumours. It may also explain why there is no close correlation as pointed out previously (Hamuro *et al.*, 1978a; Hamuro, Wagner, Röllinghoff, 1978b, c) between the capacity of distinct β(1→3) glucans to enhance *in vitro* the CTL production, to activate the APC and to enhance protective immunity to transplanted tumours.

It is not clear why previous work failed to demonstrate the impact of β(1→3) glucans on PEC mediated cytotoxicity *in vivo* and *in vitro* (Brueley-Rosette, Filorentin, Khalil & Mathé, 1976). The difference may be due to the experimental conditions or more probably to the difference in target cells used. The earlier findings describing the successful transfer of S180 regressing activity by PEC, but not by spleen, lymph node cells or serum (Maeda *et al.*, 1973) is in accordance with the results described here. Additionally, it was reported that the growth of S180 was strongly suppressed when S180 was inoculated together with PEC harvested from mice 10 days after lentinan injection (neutralization experiments) (Maeda & Chihara, 1973). On the basis of the data already published and those described here, β(1→3) glucans appear to represent a unique class of immune adjuvants. They are neutral, well characterized, purified and non-toxic substances capable of positively influencing the immunologically specific limb of T-cell mediated cytotoxic immune responses as well as the non-specific limb of macrophage mediated responses. As such they may provide a useful tool for analysing the mechanisms underlying the host-mediated resistance

against tumours and for analysing macrophage–T cell interactions during the *in vitro* generation of T-cell mediated cytotoxic immune responses.

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