Cancer Immunol Immunother (1995) 41: 389–396

ORIGINAL ARTICLE

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Prophylactic intervention in radiation-leukemia-virus-induced murine lymphoma by the biological response modifier polysaccharide K

Received: 31 July 1995 / Accepted: 26 October 1995

Abstract Polysaccharide K (PSK) is a biological response modifier used for adjuvant immunotherapy of malignant diseases. We studied the potential applicability of PSK for preventing tumor progression using an experimental model of murine lymphoma. Mice inoculated with the radiation leukemia virus (RadLV) develop thymic lymphomas after a latency of 3–6 months. However, 2 weeks after virus inoculation, prelymphoma cells can already be detected in the thymus. We found that PSK treatment induced hyperresponsiveness to concanavalin A and heightened production of interleukin-2 (IL-2) and IL-4 in spleen cells of both control and prelymphoma mice. The response was transient and was accompanied with a dominant usage of T cells expressing Vβ8, but other T cell subsets were also stimulated by PSK. T lymphoma cells expressing Vβ8.2 underwent apoptosis when incubated with PSK. Treatment of RadLV-inoculated mice with PSK delayed the onset of overt lymphoma (and mortality) but could not protect the mice from the disease. Combined treatment with PSK and a RadLV-specific immunotoxin prevented synergistically the progression of the prelymphoma cells to frank lymphoma. The results suggest that PSK contains a superantigen-like component that selectively activates Vβ8+ T cells. Its administration prelymphoma mice interfered with the process of lymphoma progression.

Key words RadLV · Lymphoma · PSK · Superantigen · Prevention

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Introduction

Polysaccharide K (PSK) is a biological response modifier (BRM), derived from *Corillus versicolor* fungi [1]. In several in vivo and in vitro experimental systems PSK was shown to potentiate humoral and cellular immune reactivities [2–8]. Its oral administration to cancer patients with colorectal or gastric carcinoma reduced morbidity and mortality with virtually no toxic or other side-effects [9–11]. Likewise, the lifespan of mice that had been inoculated with transplantable tumors or treated with carcinogens could be significantly prolonged by PSK treatment [4, 6, 8, 12–14]. The anticancer effects of PSK have been attributed to its immunomodulatory activities.

In recent years the significance of preventive, prophylactic cancer therapy has been emphasized [15, 16]. Most malignant diseases evolve through a lengthy, multistep process of tumor progression [17–19]. It was, therefore, suggested that early intervention during the latent, premalignant phases of the disease could protect against the onset of malignancy later on [20].

We have exploited a murine prelymphoma model to assess the possibility of prophylactic therapy [21–26]. Mice injected with the radiation leukemia virus (RadLV) develop thymic lymphomas after 3–6 months. However, as early as 2 weeks after virus inoculation prelymphoma cells can be detected in the thymus. Short-term treatments of the premalignant mice with a variety of chemotherapeutic agents that attack the prelymphoma cells could significantly delay and even prevent the development of lymphoma [27].

In the present study we tested the usefulness of PSK for prophylactic therapy of RadLV-induced lymphomas. We found that its administration to prelymphoma mice had immune-response-modifying activities and anti-lymphoma preventive effects.

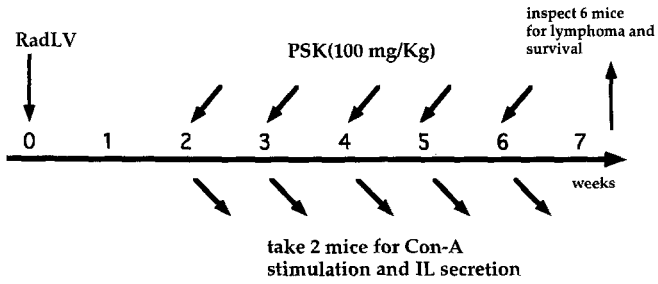

Fig. 1 Scheme of experimental protocol for the assessment of polysaccharide K (*PSK*) effect in prelymphoma. *ConA* concanavalin A, *IL* interleukin

Materials and methods

Mice

C57BL/6 female mice 6–8 weeks old were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). The mice were maintained at the SPF unit of the animal house of the Hebrew University–Hadassah Medical Center.

Virus preparation

Highly leukemogenic RadLV was harvested from cultures of 136.5 cells [28]. The virus was concentrated 100-fold by centrifuging the supernatant at 10000 *g* for 15 min then at 32000 *g* for 2.5 h at 4 °C. The pellet was resuspended in phosphate-buffered saline at 1/100th of the initial volume, divided into aliquots and stored at −70 °C.

Experimental design

To assess the effects of PSK in vivo, the experimental protocol depicted in Fig. 1 was used. Mice were inoculated i.t. with RadLV as described [29]. Control mice were sham-inoculated with saline. Beginning 2 weeks after virus inoculation, the mice were repeatedly injected i.p. with PSK (100 mg/kg). Two days after each inoculation, two mice were removed from the cage for analysis and six mice were left and observed for lymphoma development and survival. Spleen cells of two mice were tested for responsiveness to concanavalin A (ConA) as described [30]. Supernatants of 24-h splenocyte cultures were collected and their interleukin-2 (IL-2) and IL-4 content was determined using the CTLL and CT4S bioassays, respectively, as described previously [26]. In another experiment, prelymphoma mice receiving one or two injections of PSK also received an immunotoxin made of a monoclonal antibody (2F10) directed at the envelope glycoprotein of RadLV (gp70) and ricin A chain. The preparation and properties of the immunotoxin have been previously described [27, 31]. The mice were given two biweekly i.v. injections of 1 mg/kg immunotoxin during 3 weeks, six injections altogether. The mice were observed for 12 months.

Flow cytometry

T lymphocytes were detected by phycoerythrin(PE)-conjugated anti-CD3 monoclonal antibody (2C11) (Pharmingen). Expression of various Vβ families was determined with the following rat monoclonal antibodies: anti-Vβ3 (KJ-25) [32], anti-Vβ4 (KT-4) [33], anti-Vβ8.2 (F23.2) [37], anti-Vβ6 (44-28-1) [34], anti-Vβ8(1, 2, 3) (F23.1) [35], anti-Vβ8(1.2) (KJ-16) [36], anti-Vβ11 (KT-11) [38] and anti-Vβ17a (KJ-23) [39]. Reactivity of cells with the anti-Vβ antibodies was visualized with fluorescein-isothiocyanate-conjugated goat anti-(rat or hamster Ig) (Dako). Stained cells were analyzed using FACScan and Lysis II software (Becton Dickinson).

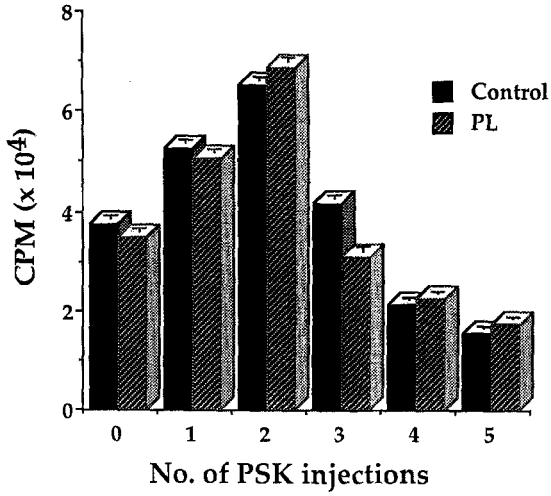

Fig. 2 Proliferative response of splenocytes from PSK-treated prelymphoma (*PL*) and control mice, stimulated in vitro with ConA. Cells (5×10^4/well) were stimulated with ConA (2.5 μg/ml) for 48 h and then pulsed for 6 h with [^{3}H]thymidine (1 μCi/ml)

Cultures

Short-term, primary cultures of T cells were maintained in RPMI medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 10 μM HEPES, 1 μM sodium pryuvate, 50 μM 2-mercaptoethanol, non-essential amino acids and antibiotics. The cells were stimulated by either ConA (5 μg/ml), IL-2 (20 U/ml) or PSK (10 μg/ml) in the presence of IL-2.

Cell depletions

Vβ8$^+$ and Vβ17a$^+$ T cells were depleted by treatment with the specific rat mAb and goat anti-(rat Ig), followed by incubation for 45 min at 37 °C with rabbit Low-tox complement (Cedar Lane).

Adherent antigen-presenting cells (APC) were removed by incubating the cells for 3 h on plastic petri dishes. Non-adherent cells were collected and washed. B lymphocyte APC were depleted using magnetic beads coated with rabbit anti-(mouse Ig).

Apoptosis

The DNA fragmentation assay of Wyllie [40] was applied. DNA extracted from treated PD1.6 lymphoma cells was separated by agarose electrophoresis in the presence of ethidium bromide. DNA fragments were visualized under UV light, to detect the "ladder" pattern typical of apoptosis.

Results

One or two injections of PSK to the prelymphoma and control mice potentiated the ability of their spleen cells to respond with proliferation to ConA (Fig. 2). However, additional PSK doses resulted in a decline in the response to normal (three injections) and even subnormal (four or five injections) proliferation levels.

The ability of the cells to secrete IL-2 and IL-4 was similarly influenced. It was elevated after one or two injections of PSK but reduced to subnormal levels as

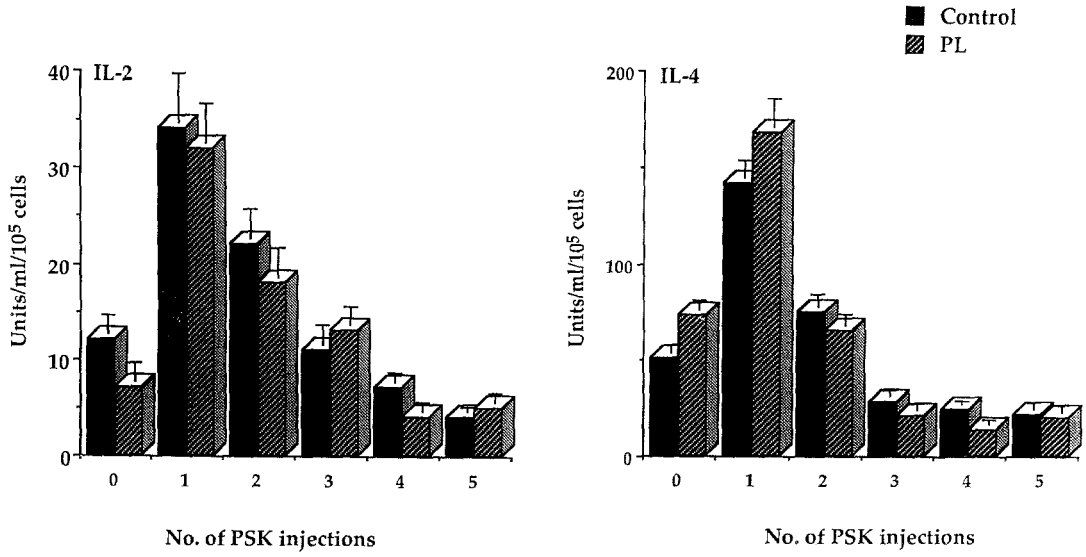

Fig. 3 Interleukin-2 (IL-2) and IL-4 secretion from splenocytes of PSK-treated prelymphoma and control mice. Cells (10^5/ml) were cultured in 24-well microplates (2 ml/well). Supernatants were collected and the content of IL-2 and IL-4 was measured by the CTLL and CT4S bioassays respectively, as described [26]

PSK administration continued (Fig. 3). Both virus-injected prelymphoma mice and control mice displayed this response pattern.

The longitudinal profile of PSK-elicited responses were reminiscent of the effect imposed on T cells expressing T cell receptor (TCR) Vβ, which is reactive with a stimulating superantigen [42]. We therefore attempted to determine whether the T cells affected by PSK belong to a particular subset or a Vβ family. PSK treatment did not change the proportion of spleen cells expressing CD4, CD8 and CD3 (results not shown). The frequencies of Vβ3$^+$, Vβ4$^+$, Vβ6$^+$, Vβ11$^+$ and Vβ17a$^+$ T cells did not change following in vitro exposure of splenocytes to either ConA, IL-2 or PSK in the presence of IL-2 (Table 1). However, the percentage of cells expressing Vβ8 fluctuated if PSK was present in the culture. It increased after 1–2 days of PSK treatment but then decreased to normal and subnormal proportions (Table 1, Fig. 4). No fluctuations in the fre-

Table 1 Effect of concanavalin A (*ConA*), interleukin-2 (*IL-2*) and polysaccharide K (*PSK*) on Vβ expression among splenic T cells. Cells were incubated with ConA (5 μg/ml), IL-2 (20 U/ml) or PSK (10 μg/ml) together with IL-2 (20 U/ml). Samples were removed daily and the frequency of cells expressing various Vβ families was determined by flow cytometry

Antibody	Specificity	Treatment	Positive cells (%)					
			Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
KJ-25	Vβ3	ConA	12±1.1	13±1.0	12±1.1	11±0.9	9±8	13±1.1
		IL-2	12±1.1	12±1.2	11±1.0	7±0.5	11±1.2	10±1.0
		PSK+IL-2	12±1.1	11±1.2	10±0.8	5±0.5	10±1.1	9±1.0
KT-4	Vβ4	ConA	6±0.5	8±0.6	6±0.5	8±0.6	5±0.5	6±0.7
		IL-2	6±0.5	6±0.5	5±0.4	8±0.8	4±0.5	6±0.6
		PSK+IL-2	6±0.5	6±0.6	4±0.5	7±0.8	5±0.4	7±0.5
44-28-1	Vβ6	ConA	3±0.4	5±0.3	3±0.3	4±0.3	4±0.3	3±0.4
		IL-2	3±0.4	4±0.3	3±0.2	4±0.4	3±0.2	5±0.4
		PSK+IL-2	3±0.4	4±0.4	5±0.4	4±0.3	4±0.3	3±0.3
F23.1	Vβ8(1,2,3)	ConA	15±1.2	16±1.4	17±1.5	17±1.5	13±1.1	11±1.0
		IL-2	15±1.2	15±1.3	15±1.6	16±1.7	14±1.5	15±1.2
		PSK+IL-2	15±1.2	31±2.5	27±2.4	13±1.4	7±0.8	3±0.3
KJ-16	Vβ8(1,2)	ConA	11±0.9	15±1.2	15±1.2	13±1.2	10±0.9	12±1.1
		IL-2	11±0.9	13±1.5	10±0.8	15±1.3	14±1.1	12±1.2
		PSK+IL-2	11±0.9	20±1.8	17±1.5	10±1.0	6±0.7	3±0.1
KT-11	Vβ11	ConA	10±0.7	14±1.3	15±1.6	10±1.1	15±1.5	9±0.8
		IL-2	10±0.7	10±0.9	11±0.9	14±1.2	9±1.0	7±0.8
		PSK+IL-2	10±0.7	10±0.7	9±0.7	11±1.2	10±1.0	11±1.0
KJ-23	Vβ17a	ConA	5±0.5	6±0.4	5±0.5	4±0.5	4±0.5	3±0.1
		IL-2	5±0.5	4±0.4	5±0.3	7±0.6	6±0.5	4±0.2
		PSK+IL-2	5±0.5	4±0.5	6±0.4	6±0.7	4±0.5	3±0.2

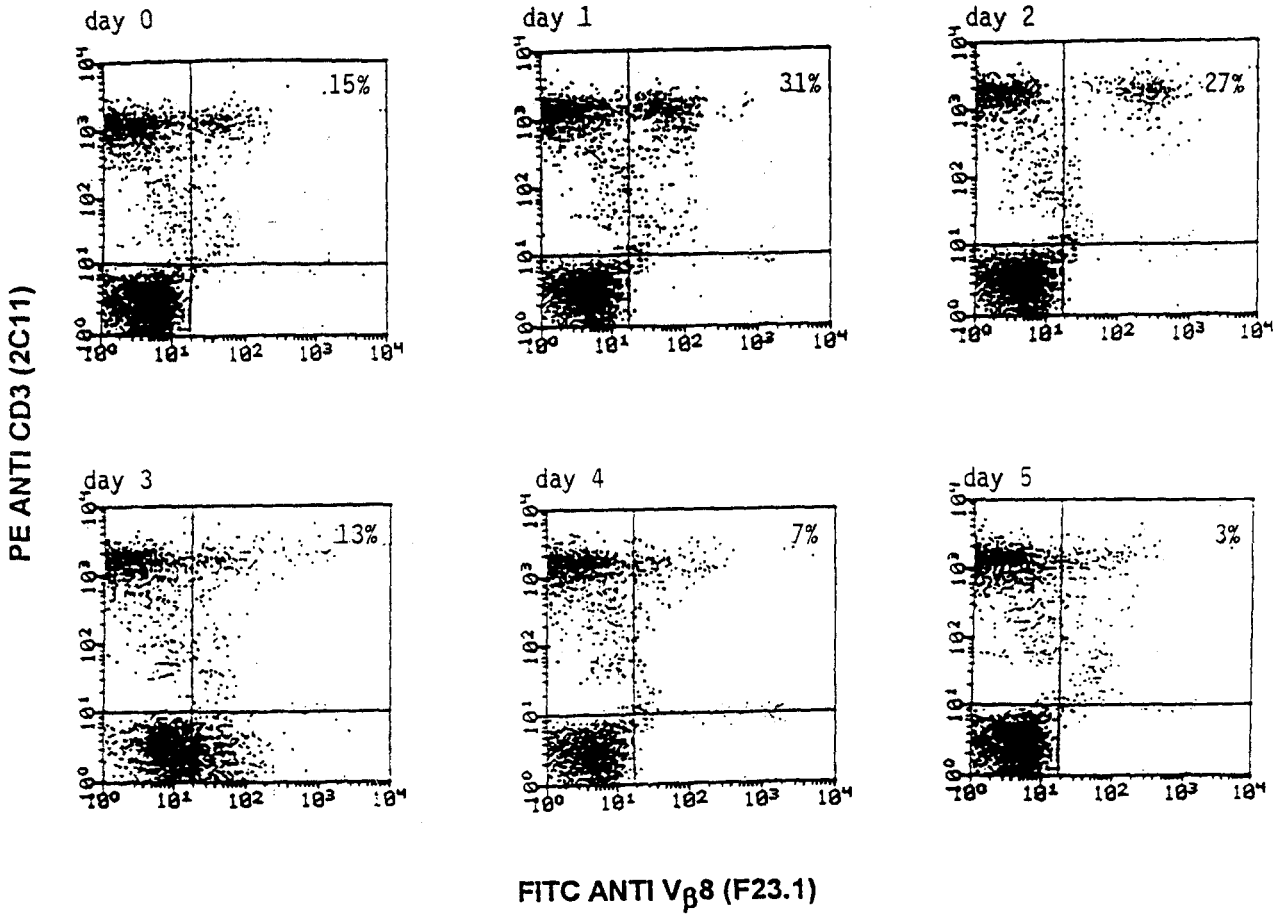

Fig. 4 Vβ8 usage following treatment of spleen cells with PSK in vitro. The percentage of Vβ8+ T cells is indicated in the *upper right box* of each dot plot

quency of Vβ8+ cells occurred in the cultures containing ConA or IL-2 only.

To find out whether Vβ8+ T cells were selectively influenced by PSK stimulation, these were depleted by treatment with anti-Vβ8 antibodies and complement. Depletion of Vβ8+ cells reduced the magnitude of the PSK response by only 18% (Table 2). For comparison, depletion of Vβ17a+ cells, the frequency of which was not changed following PSK treatment (Table 1), resulted in a 12% reduction of the PSK responsiveness. Stimulation by ConA or by IL-2 in the absence of PSK was not affected by Vβ8+ T cell depletion either. These results suggested that T cells expressing TCR with Vβ families other than Vβ8 were also stimulated by PSK, although their relative frequencies were not changed.

Since T cells are stimulated by a superantigen only when presented by APC, we examined the effect of APC depletion on the response to PSK. In the absence of APC, the frequencies of Vβ8+ cells were not affected by PSK. The proliferative response was not abrogated but it was slightly reduced (by less than 10%) (Table 3). Hence, T cells expressing Vβ families other than Vβ8 were also stimulated by PSK, presentation by APC not being required for their triggering.

To find out whether PSK can directly affect thymic lymphoma cells expressing Vβ8, we tested its effect in the Vβ8-expressing PD 1.6 lymphoma cells (Fig. 5). Incubation with PSK at 10 μg/ml inhibited the proliferation of PD 1.6 cells (results not shown). Electrophoresis of DNA digested from PSK-treated PD 1.6 cells revealed fragmentation into multimers of 180–200 base pairs, displayed as a "ladder" (Fig. 6). This result suggests that PSK induced the PD 1.6 lymphoma cells to undergo apoptotic death. The growth of the Vβ8 RadLV-induced lymphomas PD 1.6 (Vβ4) and B10 (Vβ17a) was not affected by PSK (results not shown).

Table 2 Effect of ConA, IL-2 and PSK on the proliferation of various T-cell subsets

Cells	[^{3}H]Thymidine incorporation (cpm)			
	No treatment	ConA	IL-2	IL-2+PSK
Total	647±52	53760±4282	1243±156	6584±591
Vβ8-depleted	508±47	39406±3604	1140±107	5370±488
Vβ17a-depleted	491±44	45082±4633	1226±148	5807±510

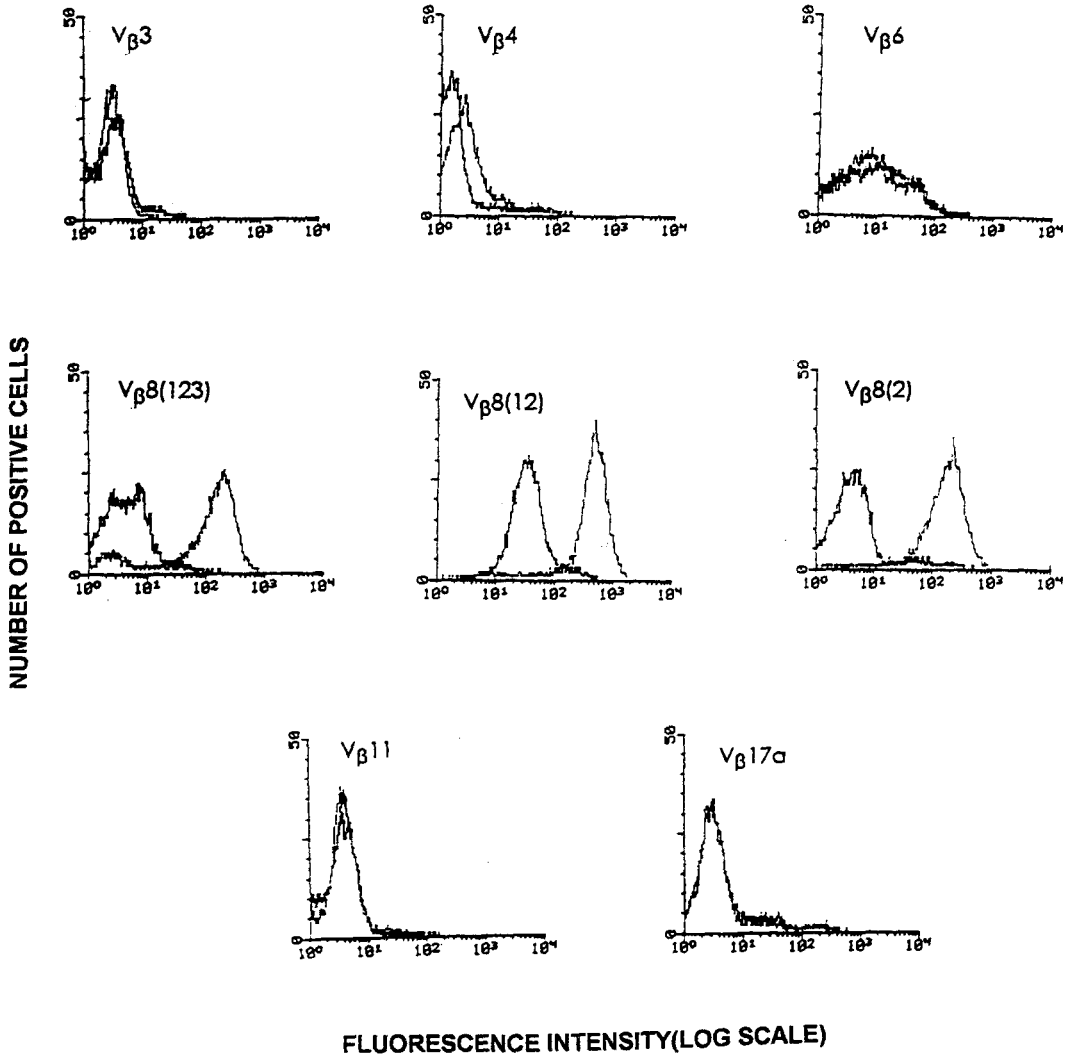

Fig. 5 Vβ typing of PD 1.6 lymphoma cells. Monoclonal antibodies used for detection of Vβ specificities are listed in Materials and methods

Table 3 Stimulation of macrophage and B-cell-depleted splenocytes by PSK in the presence of IL-2. Vβ8 frequency was determined by the F23.1 antibody

Spleen cells	Proliferation	Vβ8 frequency (%) after:	
		1 day	4 days
Total	24756±2582	28±1.9	7±0.5
Macrophage- and B-cell-depleted	22437±1920	17±1.6	16±2.3

Table 4 PSK delays radiation-leukemia-virus-induced leukemogenesis. PSK was administered i.p. once a week and the monitoring of lymphoma development was terminated at 12 months. Significance was estimated by the log-rank test

No. of PSK injections	Lymphomas: no./total	Survival (days)		Significance *P*
		Median	Range	
0	6/6	108	83– 131	
1	5/6	141	119–>365	<0.005
2	5/6	148	110–>365	<0.005
3	6/6	125	87– 248	<0.01
4	5/6	112	87–>365	NS
5	6/6	115	83– 138	NS

The effect of PSK treatment on lymphoma development and mouse survival is summarized in Tables 4 and 5. One or two injections of PSK early during the premalignant latency delayed lymphoma development by 5–6 weeks (Table 4). Additional PSK injections did not improve the effect but rather reduced the life span of the animals. PSK treatment did not prevent lymphoma development.

We have previously shown that immunotoxins of anti-RadLV antibodies coupled to ricin A chain could delay lymphoma development when administered to prelymphoma mice [27] (and Table 5). An optimal effect was achieved with immunotoxin given by six i.v. injections during 3 weeks. Immunotoxin treatment and one or two weekly injections of PSK had a synergistic antitumor effect (Table 5). The majority of mice remained tumor-free and the few mice that developed tumors died with considerable delay. This experiment was repeated twice with similar results.

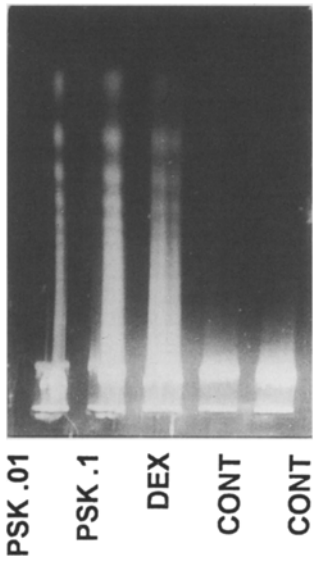

Fig. 6 PSK-induced DNA fragmentation in PD 1.6 cells. Lanes (*from left to right*) display DNA prepared from control untreated cells, control, ConA-treated cells, dexamethasone(0.1 μM)-treated cells (positive control), PSK(0.1 mg/ml)-treated cells, PSK(0.01 mg/ml)-treated cells

Discussion

PSK is a multifactorial BRM, affecting various compartments of the immune response. In a previous paper, we demonstrated two immunopotentiating activities of PSK in mixed lymphocyte/tumor cultures [43]. PSK enhanced specific T cell reactivities against syngeneic, RadLV-induced lymphoma and abrogated immunosuppression mediated by RadLV-primed lymphocytes. As a follow-up of that in vitro study, we sought to find out whether PSK could be applied for prophylactic intervention in RadLV-induced leukemogenesis. To this end, we first injected PSK into virus-inoculated and control mice, and evaluated its effect on various T cell immune functions. Both the mitogenic responses and lymphokine production were transiently enhanced by PSK treatment in vivo, with no difference between prelymphoma and control mice. Surprisingly, after the strong boost occurring after 1–2 weeks of PSK injections, the T cell responsiveness was rapidly reduced and then suppressed as the exposure to PSK continued. Hence, contrary to other BRM, which overstimulate T cell responses and cytokine production [44–46], PSK elicits a balanced cytokine response that peaks early during PSK treatment but then gradually declines. This finding is potentially significant when clinical application of PSK is considered. A major problem of adjuvant immunotherapy with BRM is the continuous hyperstimulation of cytokine production, resulting in vascular and cardiac toxicities [46–49]. It may be expected that the regulated secretion of lymphokines induced by PSK will reduce the side-effects of long-term treatment.

The fluctuations in the response of spleen cells to PSK suggested that PSK might contain a superantigen-like component. Superantigens can engage a large number of T cell clones expressing a particular family of Vβ, and therefore induce a vigorous T cell response. This response is, however, transient because chronic exposure to a superantigen results in apoptosis of the responding T cells, which will be gradually eliminated [32, 42]. On the basis of these considerations, we began searching for a Vβ family that might be preferentially triggered by PSK. Out of six Vβ specificities, defined by monoclonal antibodies, only the frequency of Vβ8-positive cells fluctuated following stimulation with PSK. It increased about twofold shortly after exposure to PSK, but decreased to subnormal levels within 3–5 days. This response pattern was not observed when the T cells were activated by mitogen or when other Vβ families were analyzed. We therefore concluded that PSK affects T cells expressing TCR with Vβ8.

Next we asked whether Vβ usage is the only activity imposed on T cells by PSK. Since PSK is an extract containing an array of fungus-derived proteins and saccharides, it could be expected to interact with various T cell subsets and affect multiple T cell functions. Indeed, removal of Vβ8$^+$ cells did not result in a drastic loss of responsiveness to PSK, indicating that other T lymphocytes responded as well. Moreover, since the activity of a superantigen is dependent on antigen presentation, we examined the impact of APC depletion on stimulation by PSK. In the absence of APC, the proportion of Vβ8$^+$ cells was not affected by PSK. However, the remaining T cells were stimulated by PSK in what appeared to be a non-superantigen-like activity. Hence, Vβ usage is yet another activity of PSK that is mediated by a superantigen-like component that remains to be defined.

Table 5 Administration of both PSK and ricin-A–anti-gp70 immunotoxin (*IT*) prevents lymphoma development. The experiment was terminated at 12 months. PSK was administered i.p. once a week, IT was administered i.v. twice a week. Significance was estimated by the log-rank test

Number of injections		Lymphomas:	Survival (days)		Significance
IT	PSK	no./total	Median	Range	*P*
0	0	6/6	112	85– 140	
6	0	6/6	137	94– 149	<0.005
0	1	6/6	146	107– 153	<0.005
0	2	5/6	142	104–>365	<0.005
6	1	1/6	>365	132–>365	<0.001
6	2	2/6	>365	130–>365	<0.001

Since superantigens induce apoptotic death in T cells bearing a reactive Vβ, we asked whether PSK could induce direct apoptosis of the Vβ8+ lymphoma PD 1.6. Incubation of PD 1.6 cells with PSK inhibited the growth of the cells and induced them to apoptose. Hence, an additional feature of PSK is its ability to induce the apoptotic death of Vβ8+ malignant T cells.

An important goal of our study was to test whether PSK could protect prelymphoma mice from lymphoma development when given during the latency of the disease. When administered alone, the PSK treatment postponed the onset of lymphoma and the death of the mice by 5–6 weeks. Thus, the biological-response-modifying activities of PSK demonstrated in vitro are, apparently, effective in providing protective immunity against lymphoma development in vivo. The most notable response was registered after one or two injections of PSK, corresponding to the transient activation of T cells by the drug. These results suggested that the effectiveness of PSK as a BRM is dose-dependent and that short-term treatments are likely to yield stronger antitumor effects in vivo.

Although PSK significantly prolonged survival, it did not prevent lymphoma development. In a previous study, we demonstrated that a RadLV-specific immunotoxin could synergize with cyclosporin A in inhibiting the outbreak of primary lymphomas induced by RadLV [27]. Since the inhibitory effects of PSK and the immunotoxin are induced by entirely different mechanisms (the former is an immunopotentiator and the latter targets and kills prelymphoma cells), we assumed that their combination may provide synergistic protection to prelymphoma mice. Indeed, when administered together at an optimal dose and timing, PSK and the immunotoxin provided complete protection against lymphoma development to the majority of the mice. These results indicate that when a large population of prelymphoma cells is directly eliminated by an immunotoxin, the progression of the remaining, potentially malignant cells to frank lymphoma can be prevented by potentiating host immunity with PSK.

It is noteworthy in this regard that, whereas an effective regimen for immunotoxin treatment required six consecutive injections, the optimal dose of PSK was a single injection, given more than 10 weeks before the expected outbreak of a frank lymphoma. This observation demonstrates the effectiveness of PSK in prophylactic intervention during tumor progression, which apparently potentiates simultaneously multiple antitumor immune reactivities.

The benefits of PSK for adjuvant immunotherapy of malignant diseases has been documented in the past [9, 10, 11]. Considering the low drug doses required for prophylactic protection and the negligible side-effects that they produce, the efficacy of PSK in preventive therapy should be assessed for cancers that are effectively controlled by BRM and with a premalignant phase that can be accurately identified.

Acknowledgements We gratefully acknowledge the support of the Concern Foundation of Los Angeles, the Israel Science Foundation, the Israel Cancer Research Fund and the Swedish Cancer Society. We thank Ms. Yaakovah Jacobs for her skillful secretarial assistance.

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