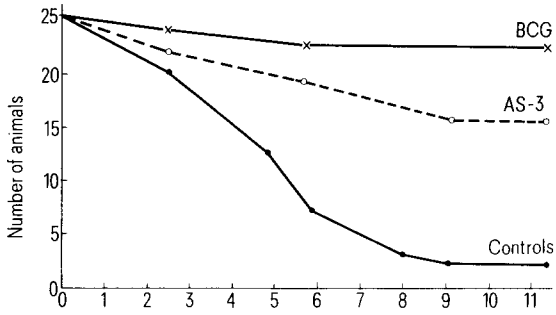
neutralizing antibodies against vaccinia virus using a 50% plaque reduction in HeLa-cells.

Results and discussion. No side effects of BCG or of any other immunostimulant were observed. No drug protected the animals against i. cer. infections. Controls as well as treated animals appeared well for 2–3 days, then they became lethargic, lost appetite and developed ruffled hair. 3–4 days later they died. The mortality in all groups was 98–100%.

After i.p. infections 90% of the controls succumbed to the infection. Levamisole, Isoprinosine, Echinacin or BM 12.531 had no effect upon the course of the disease in this model. After prophylactic treatment with AS-3, 75% of the animals survived. When AS-3 was started after the infection, the survival rate was 35%. In the group, which received *C. parvum* prior to the infection, 60% of the animals survived. No effect was seen, if this drug was started after infection.

Pretreatment with BCG yielded a survival rate of 90%. There were no differences between those groups which received BCG 7 or 12 days prior to the infection (figure). The oral vaccination with BCG, however, had no effect. 20 surviving mice received an i. cer. challenge with vaccinia virus Mal 30 days after the primary infection. Wheras all aged-matched controls died, only 3 mice from the BCG group succumbed to the infection. Animals which survived the primary infection after pretreatment with BCG developed titers of neutralizing antibodies against vaccinia virus between 1:128 and 1:512. No titers were found in control animals.



Effect of BCG (×——×) and AS-3 (○——○) upon the course of the i.p. infection of NMRI-mice with vaccinia virus Mal, in comparison to untreated controls (●——●). Number of surviving animals: BCG 23/25; AS-3 18/25; controls 2/25. No further mortality after day 11 upto the end of the experiment (day 30).

Our results confirm previous reports that BCG given prior to an infection increases the resistance of animals against infections with DNS-viruses[4–7]. *C. parvum* exerts some effect if applied before the infection. The findings that AS-3 increases the survival rate, especially if it is given prior to the infection, is interesting and needs further elucidation. Why neither BCG nor any other drug protected the animals against i. cer. infection is open for discussion. Probably the course of the disease after an i. cer. infection is too rapid, and the immunostimulant might not increase the host-resistance within this short period of time. As BCG acts through activation of macrophages[12,13], it is conceivable that the macrophages do not protect against i. cer. infections, as they do not cross the blood-brain barrier. BCG treatment in patients with cancer might exert a positive effect against opportunistic infections[14], besides the increase produced in nonspecific resistance against the tumor.

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PPD-induced blastogenesis is auto-regulated by suppressor cells generated in vitro[1]

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Summary. Suppressor cell induction can be demonstrated during antigen specific blastogenesis by using the same methods which have shown induction of suppressor cells by Con A. Since suppressor cells are rapidly generated during antigen specific blastogenesis, they must regulate the final level of blastogenesis induced during the seven day in vitro incubation.

Regulation of a variety of immune phenomena by suppressor cells is now well established[2,3]. The inhibitory effect of suppressor cells is usually detected by mixing them with fresh cells capable of a given immune response. When the immune response of the mixed population is less than that of a control, it is supposed that suppressor cells are included in the added cells. Thus Shou et al.[4] and Hubert et al.[5] have reported human suppressor cells which are activated by preincubation with concanavalin A (Con A) and inhibit subsequent blastogenesis of syngeneic peripheral mononuclear cells (PMC) stimulated with Con A as well as other mitogens. The detection of Con A-induced suppressor activity in PMC is now a useful method for monitoring potential suppressor cell function in patients with autoimmune diseases[6,7]. However the question whether such generation of suppressor cells is a general phenomenon accompanied by a lymphoproliferative response is still uncertain. We report here that a specific antigen, purified tuberculo-

protein derivative (PPD) can also definitely, although not so strongly as Con A, induce suppressor cells during in vitro culture of PMC derived from subjects who have been naturally sensitized to the protein.

Material and methods. PMC were separated on Ficoll-Paque (Pharmacia) from heparinized blood and cultured in F-10 culture medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) at 37 °C in 5% CO_2 in moist air. For the studies of PPD-induced suppressor cell activity, PMC suspension (8×10^5/ml) obtained from each individual shown in the figure was incubated with or without PPD (Parke-Davis) at 37 °C for 40 h (1st-step culture) followed by exposure to mitomycin C (Sigma, 50 µg/ml) for 30 min, then washed 4 times with phosphate buffered saline (PBS) and once with culture medium, each procedure with 20 vol. of the fluids. The pretreated PMC were mixed with fresh PMC obtained from the same donor to attain a cell concentration of 8×10^5/ml of each cell source. The mixture was cultured (0.2 ml per well) for another 3 days during stimulation by phytohemagglutinin-P (PHA, Difco) at a concentration of 1:1000 dilution, or for 7 days under stimulation with PPD (2.5 µg/ml) (2nd culture). Each culture was pulsed with ^{125}I-IUdR to assess DNA synthesis as described in table 1. For the studies of possible suppressor activity in culture fluids, supernatants were collected 40 h after the 1-step culture and, from each, 50 µl was added to a 2nd culture which included finally 8×10^4 fresh PMC/0.2 ml of culture medium/well. The cultures were stimulated with PHA at a concentration of 1:1000 dilution. After 3 days they were harvested to assess DNA synthesis using 4 h labelling with ^{125}I-IUdR as in table 1.

Results and discussion. The dose related response of PMC to PPD in several individuals is shown in the figure. In the culture system employed, an optimal concentration of PPD for maximal blastogenic response was at 2.5 µg/ml except for 1 subject (JB), who was superpositive in the PPD skin reaction. A good correlation was observed between the intensities of the in vitro and in vivo reactions. Individuals NA and TA were weakly and intermediately sensitized, respectively, whereas EM and JB were strongly sensitized. 1 individual (CD), who was negative in the skin reaction, was absolutely lacking in blastogenic response to PPD at every dose level. For all of these subjects, inducibility of suppressor cells in PMC was examined after preincubation of PMC with various doses of PPD. Typical results showing evidence of in vitro generation of suppressor cells were obtained using PMC derived from EM (table 1). It is clear from the results that PPD-pretreated PMC suppress the blastogenic response of the 2nd cultures which were challenged with PHA as well as with PPD, whereas no such suppression was observed after OVA-pretreatment. Dose-related suppression was noted following the doses of PPD used for the 1st-step culture which coincided with the gradual effective doses for blastogenesis, as shown in table 1. The most remarkable suppression was found when PMC were pretreated with 2.5 µg/ml of PPD, which was the optimal dose for the blastogenic response of PMC derived from the same subject. It should be emphasized that blastogenic response observed in the 2nd culture was attributable to untreated responder lymphocytes, because PPD-pretreated PMC had been treated with mytomycin C (MC) at the end of pretreatment to stop further DNA synthesis. Although no blastogenesis was observed in the cultures composed of such MC-treated cells alone, even after stimulation with PHA or PPD (data not shown), an increase of DNA synthesis was found without adding

Table 1. Effects of PPD-pretreated PMC on 2nd cultures stimulated with PHA and PPD

Subject, EM	Protein concentration (µg/ml) in 1st-step culture					
2nd culture: stimulated with	No PPD (control)	PPD 0.0025	PPD 0.025	PPD 0.25	PPD 2.5	OVA 2.5
1. PHA, 1:1000	3023±290*	2950±431	2895±301	2517±150	2064±285	2919±274
None	61±20	61±38	60±23	88±54	134±38	58±34
Net response	2962±290	2889±431	2835±301	2429±150	1930±285	2861±274
		NS**	NS	$p<0.05$	$p<0.02$	NS
2. PPD, 2.5 µg/ml	1136±121	1209±193	1064±243	868±160	745±74	1227±159
None	81±39	60±10	96±32	121±36	442±41	83±25
Net response	1055±121	1149±193	968±243	747±160	303±74	1144±159
		NS	NS	$p<0.05$	$p<0.001$	NS
Subject, CD						
1. PHA, 1:1000	7922±520*		7945±678	8081±770	8168±474	8097±552
None	23±6		26±8	31±10	20±8	28±11
Net response	7899±520		7919±678	8050±770	8148±474	8069±552
			NS**	NS	NS	NS
2. PPD, 2.5 µg/ml	32±19		30±14	35±28	31±26	33±11
None	26±3		31±15	32±5	29±6	30±12

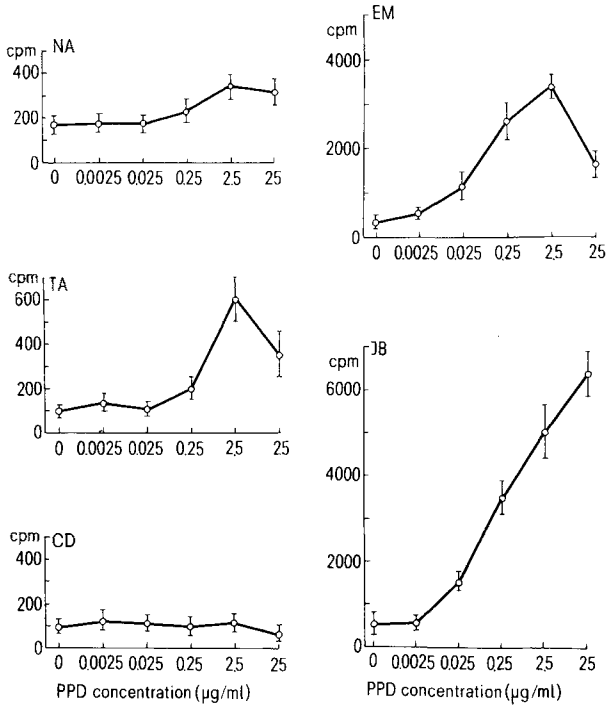
1. 3-day sulture pulsed with 0.5 µCi of ^{125}I-IUdR during the last 4 h. 2. 7-day culture pulsed with 0.5 µCi of ^{125}I-IUdR during the last 20 h.
* CPM (mean ± SD); ** Comparison to control (culture medium); NS: not significant; OVA: ovalbumin (Sigma).

Table 2. Effects of supernatants of PPD-treated 1st-step cultures on fresh PMC culture stimulated with PHA

Subject, EM	Protein concentration (µg/ml) in 1st-step culture					
2nd culture (fresh PMC): stimulated with	No PPD (control)	PPD 0.0025	PPD 0.025	PPD 0.25	PPD 2.5	OVA 2.5
PHA, 1:1000	2681±161*	2775±258	2695±256	2617±151	2628±59	2599±357
None	20±6	29±19	23±17	32±10	73±13	28±15
Net response	2661±161	2746±258	2672±256	2585±151	2555±59	2571±357
		NS**	NS	NS	NS	NS

* CPM (mean ± SD); ** Comparison to control (culture medium); NS: not significant; OVA: ovalbumin (Sigma).

stimulants in the 2nd cultures composed of equal numbers of fresh and MC-treated PPD-pretreated PMC (table 1). This increment was slight on the 3rd day and greater on the 7th day of culture when potent doses of PPD had been used in the 1st-step culture. This is possibly due to carryover of the antigen, presumably trapped (or processed) by macrophages in the 1st-step culture leading to the activation of responding lymphocytes in the 2nd culture. 5 washings of the pretreated PMC with saline or with culture medium (each washing with 20 vol. of the fluids) could not prevent the augmentation of baseline-blastogenesis in the 2nd culture. We do not yet have a reagent comparable to the α-methyl-D-mannoside used in the Con A-induced suppressor assay, which can completely remove residual Con A from the 1-step culture. The results obtained from the subject, CD, who was absolutely not sensitized to PPD, showed no induction of suppressor activity in the 1st-step culture of PMC (table 1). When blastogenic response of PMC to PPD was lacking as in this subject, it was impossible to induce specific suppressor activity. Similar results to the subject, EM, were obtained from other individuals JB, TA, and NA, who were all sensitized to PPD (data not shown). JB was most remarkable, since PPD-induced suppressor activity was significantly detected ($p < 0.05$ and $p < 0.01$ for PHA and PPD challenges respectively), even in the assay using a lower concentration of PPD (0.025 µg/ml) for the 1st-step culture. Although it was always possible in repeated experiments on the PPD-sensitized individuals to detect the nonspecific suppressor cells which reduced PHA-stimulated blastogenesis in the 2nd culture, the specific suppressive effect to PPD challenge by PPD-induced suppressor cells was sometimes hard to detect because of low CPM in the 2nd culture when low responders to the protein (such as NA) were examined. Thus it should be noted that suppressor activity induced with PPD in the 1st-step culture is quite dependent on the state of previous sensitization of individuals to the protein, that there is a dose related induction of suppressor activity dependent upon the concentration of PPD used for the 1st-step culture, and that suppressor activity once induced with PPD works both specifically and non-specifically on further blastogenic response. Another experiment with shorter intervals for PPD pretreatment showed that after 24 h of pretreatment it was also possible to demonstrate suppressor cells, but not so dramatically as in the experiments with 40 h pretreatment (data not shown). In order to clarify if suppressive activity is released into the culture supernatant of the 1st-step culture, the supernatant was added to fresh PMC culture simultaneously with PHA stimulation (table 2). It is clear that no such activity as observed in table 1 was detected using the supernatant in the system. Several reports have shown in vitro induced suppressor cells in the mixed lymphocyte culture in murine models[8-12]; some of the suppressor cells are cytotoxic and others are not. In our system, cytotoxicity does not seem to have much influence on the final results, since cell viability in the 2nd cultures (without adding stimulants) never showed a significant difference between control and PPD-treated (2.5 µg/ml) cultures (e.g. 86.3 ± 5.5 and 88.7 ± 4.0% at day 3 of culture, control and PPD-treated cultures respectively for the subject, EM). In vitro generation of antigen specific suppressor cells which regulate anti-body production has been reported in mice using sheep red cells[13] or keyhole hemocyanin[14] to treat virgin lymphoid cells. The suppressor cells described in the present paper are different from these in the sense that they are not generated from virgin PMC. Our observation shows that the antigen specific blastogenesis is auto-regulated by suppressor cells generated during the in vitro blastogenic response. The generated suppressor cells might affect the expression of blastogenic response of antigen specific culture systems, since it usually takes 7 days or more to complete the tests. In addition, the present data suggest that PPD-induced suppressor cells might have untoward activities in cancer patients treated with PPD-immunotherapy when they have been already sensitized to the protein.



Dose-related blastogenic response to PPD in PMC cultures derived from several subjects. The cultures were performed in quadruplicate in microculture plates (Falcon), each well containing 8×10^4 cells in 0.2 ml of culture medium with or without purified tuberculoprotein derivatives (PPD, Parke-Davis). At termination of culture, cells were pulsed with ^{125}I-iododeoxyuridine (^{125}I-IUdR, NEN, 0.5 µCi per well) for the last 20 h of culture and harvested on glass wool filters using an automated cell harvester. Incorporation of ^{125}I-IUdR into DNA was measured by using a gamma counter. The data are expressed as mean ± SD.

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