Immunology 1982 **47** 503

Regulation of immune responses against the syngeneic ADJ-PC-5 plasmacytoma in BALB/c mice

III. INDUCTION OF SPECIFIC T SUPPRESSOR CELLS TO THE BALB/C PLASMACYTOMA ADJ-PC-5 DURING EARLY STAGES OF TUMORIGENESIS

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Accepted for publication 10 May 1982

Summary. Initial stages of tumour growth are not easily accessible to investigation. Therefore an experimental procedure was developed to mimic tumorigenesis as closely as possible. BALB/c mice received intraperitoneally exponentially increasing numbers of irradiated syngeneic ADJ-PC-5 plasmacytoma cells. The initial injection began with two cells per mouse and according to the generation time of this tumour, subsequent doses were doubled until mice had received up to 10^5 tumour cells. At various stages of treatment, peritoneal exudate cells (PEC) and spleen cells (SC) were tested for either cytotoxicity or specific suppression of induction of a primary *in vitro* T-cell cytotoxic response (CTL) of BALB/c spleen cells against ADJ-PC-5 plasmacytoma cells. No cytotoxic PEC were found. Instead, PEC from mice in which the final tumour cells number had reached or exceeded 10^3 irradiated ADJ-PC-5 cells, induced complete suppression of this primary *in vitro* CTL. Specificity was found both in the induction and effector phase of suppression. Specific suppression was mediated by Thy-1.2^+ cells and amplified by non-specific suppression through adherent cells. The data are discussed in context with previous findings on the *in vivo* immunogenicity and tolerogenicity of the ADJ-PC-5 plasmacytoma. They suggest that induction of T suppressor (Ts) cells might be an early event in tumorigenesis.

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0019-2805/82/1100-0503$02.00

INTRODUCTION

Tumours arising spontaneously in experimental animals are not accessible to analysis until they become manifest at rather late states of tumorigenesis. Early, possibly critical phases remain obscured due to difficulties in detecting and manipulating microfoci of spontaneous tumours. Therefore transplantable tumours have been used widely to gain insight into the host anti-tumour reactivity.

Transplantable tumours grow progressively in their syngeneic hosts. This could be due to lack of antigenicity. However, in cases where immunogenicity has been demonstrated (Potter & Walters, 1973; Röllinghoff, Rouse & Warner, 1973; Mc Coy, Dean, Law, Williams, Mc Coy & Holiman, 1974; Burton & Warner, 1977) escape mechanisms exist to explain progressive growth. It can be argued that transplantable tumours have overridden a barrier of natural killer (NK) cells (Collins, Patek & Cohn,

1981). This would imply that NK cells usually play a dominant role in surveillance mechanisms (Kärre, Klein, Kiessling, Klein & Roder, 1980; Talmadge, Meyers, Prieur & Starkey, 1980) and that the classical immune system is rather inefficient in rejecting tumours. However, it is equally conceivable that tumours are immunogenic and that the immune system in those tumour-bearing animals is paralysed by intrinsic regulatory mechanisms of the system itself: Shed antigen (Alexander, 1975), enhancing antibodies (Kaliss, 1958) or inhibitory immune complexes (Sjögren, Hellström, Bansel & Hellström, 1971) must be considered to interfere with immunological defence mechanisms. Furthermore, a possible function of cytotoxic T cells could be counteracted by suppressor cells (Fujimoto, Greene & Sehon, 1976a; Cihak, Ziegler & Kölsch, 1981b).

It is difficult to apply results from experiments with transplantable tumours to the situation of spontaneous tumorigenesis, since spontaneous tumours often seem to have little or no immunogenicity (Hewitt, Blake & Walder, 1976; Klein & Klein, 1977).

In order to gain some insight into the immune status of tumour-bearing animals during early phases of tumorigenesis one cannot avoid the use of transplantable tumours. Experimental findings might be relevant if situations can be chosen which have some parameters in common with spontaneous tumorigenesis. Our approach uses an exponential increase of the antigenic load of tumour cells in an animal as the closest approximation to the situation in clonal tumour expansion and monitors the hosts immune response at various stages. The experiments to be reported suggest that potential immune reactivity against a tumour might be undermined by specific Ts cells in an inital phase of tumorigenesis when the antigenic load is still too low to induce protective immunity.

MATERIALS AND METHODS

Mice

BALB/c female mice (obtained either from Olac, Shaws Farm, Bicester, England or from Bomholtgard, Ry, Denmark), 8–16 weeks old, were used in the experiments.

Tumours

The non-secreting variant (NS) of BALB/c plasmacytoma ADJ-PC-5, originally obtained from Dr J. Haimovich, Weizmann Institute, Rehovot, Israel, was used as a model tumour (Blatt & Haimovich, 1977). The generation time of ADJ-PC-5 was about 24 hr. The C57Bl/6 T cell lymphoma EL4 and the DBA/2 mastocytoma P815 were used in specificity controls. Tumours were maintained by serial passages in tissue culture in Dulbecco's modified Eagle's medium (DME) containing 10% foetal calf serum (FCS).

In vitro *T-cell cytotoxicity*

Primary syngeneic BALB/c anti-ADJ-PC-5 T-cell cytotoxicity was induced *in vitro* by cultivating 2×10^7 normal BALB/c spleen cells together with 2×10^6 ADJ-PC-5 cells (X-irradiated with 4000 rad) in Dulbecco's modified Eagle's medium supplemented with non-essential aminoacids, 10% foetal calf serum, 5×10^{-5} M 2-mercaptoethanol and 2×10^{-3} M l-glutamine, 100 u. penicillin/ml and 100 μg streptomycin/ml (MLC medium). T-cell cytotoxicity was tested after 7–8 days of culture in a 6 hr ^{51}Cr-release assay at various effector:target cell (E:T) ratios using ^{51}Cr-labelled ADJ-PC-5 cells as targets. The percentage specific ^{51}Cr-release was determined as described by Brunner, Mauel, Cerottini & Chapius (1968). A primary BALB/c anti-C57Bl/6 T-cell response was induced by co-cultivating 2×10^7 BALB/c spleen cells with 2×10^7 C57Bl/6 spleen cells (X-irradiated with 2000 rad). In this case E14 lymphoma cells were used as target cells in the ^{51}Cr-release assay.

In vivo *induction of suppressor cells*

Groups of BALB/c mice were injected intraperitoneally with increasing numbers of 4000 rad irradiated syngeneic ADJ-PC-5 cells. The experimental protocol of injecting graded numbers of irradiated tumour cells into animals of the various groups is depicted in Fig. 3. Control animals used in suppression experiments were either untreated or had received EL4 lymphoma cells according to the above protocol.

In vitro *test for suppression and characterization of suppressor cells*

PEC or SC (5×10^6) of mice of various groups were each added to primary mixed lymphocyte tumour reactions at day 0 of culture and the cells co-cultivated for 7–8 days.

Plastic adherent cells were separated from non-adherent cells by incubating 1×10^7 PEC for 20 hr in 15 ml of MLC medium in a 50 ml tissue culture flask. After this time non-adherent cells were harvested with a Pasteur pipette. Their suppressive activity was tested

by further co-cultivating them with a primary anti-ADJ-PC-5 *in vitro* culture. Remaining adherent cells were overlayered with fresh medium. They were further cultivated with normal BALB/c spleen cells and irradiated stimulator cells in the tissue culture flask originally used for separation.

Treatment of cells with monoclonal anti-Thy-1.2 antibody (New England Nuclear, Dreieich, F.R.G.) was done at a final antibody dilution of 1:50. Rabbit serum absorbed with agarose and with 10^8 BALB/c spleen cells was used as a source of complement at a final dilution of 1:10. PEC were incubated with anti-Thy-1.2 antibodies for 1 hr at 4°, washed four times and then further incubated for 1 hr with complement at 37° and again washed four times.

RESULTS

Primary *in vitro* T-cell cytotoxicity of BALB/c spleen cells directed against the syngeneic plasmacytoma ADJ-PC-5

A primary *in vitro* T-cell cytotoxic response of BALB/c (H-2^d) SC against the plasmacytoma ADJ-PC-5 (H-2^d) was established as a test system for ADJ-PC-5 induced suppressor cells (Fig. 1). The specificity of this *in vitro* cytotoxic T-cell response was tested using P815 (H-2^d) and E14 (H-2^b) target cells (Fig. 2). E14 cells

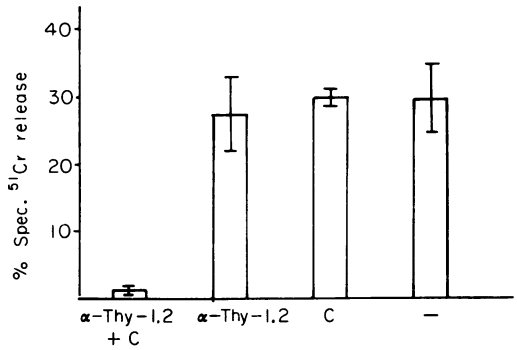

Figure 1. Primary *in vitro* T-cell cytotoxic response of BALB/c responder SC against 4000 rad irradiated syngeneic ADJ-PC-5 plasmacytoma cells after 8 days of culture. Treatment of BALB/c spleen cells with monoclonal anti-Thy-1.2 antibodies and agarose-absorbed rabbit serum as source of complement (C) abolishes their cytotoxic activity in a ^{51}Cr release assay against ADJ-PC-5 target cells. The three controls were untreated spleen cells (—) or those which were incubated with either absorbed rabbit serum (C) or monoclonal anti-Thy-1.2 antibodies (α-Thy-1.2) alone. E:T ratio was 35:1.

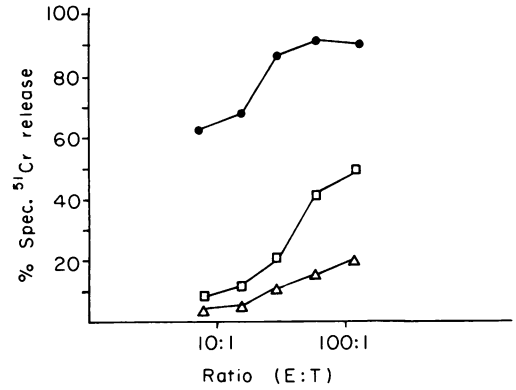

Figure 2. Specificity of the primary T-cell cytotoxic response of BALB/c (H-2^d) spleen cells against syngeneic ADJ-PC-5 (H-2^d, ●) target cells. Specificity was tested against both haplotype-indentical DBA/2 (P815, H-2^d, □) and non-identical C57Bl/6 (EL4, H-2^b, △) tumour target cells. There was no cross-reactivity against EL4 cells. A partial cross-reactivity against P815 cells was observed at high E:T cell ratios.

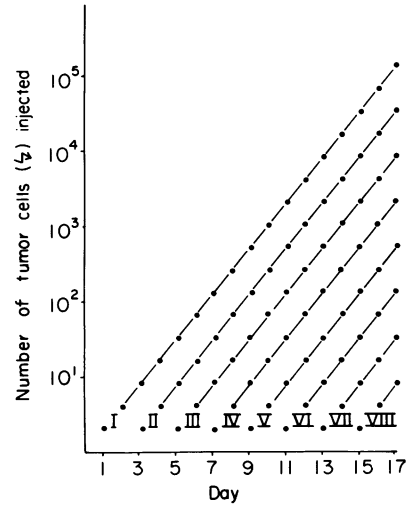

Figure 3. Experimental protocol for the simulation of early phases of tumour growth intended to mimic the antigenic load during clonal expansion of a malignant cell. BALB/c mice were divided into eight groups consisting of four mice each. Starting with two (4000 rad irradiated) ADJ-PC-5 plasmacytoma cells, mice received an exponentially increasing number of irradiated ADJ-PC-5 cells every day (according to the *in vivo* and *in vitro* doubling time of the tumour). Injections of mice of the different groups started at different days. Thus on day 17 animals were available in which accumulation of ADJ-PC-5 cells ranged from eight cells (group VIII) to 10^5 cells (group I). PEC from those animals were assayed for their suppressive effect on induction of a primary *in vitro* T-cell cytotoxic response of BALB/c SC against syngenic ADJ-PC-5 cells.

were not lysed. There is some cross-reactivity between ADJ-PC-5 and P815 target cells detectable at high E:T ratios. It is probably due to a common viral antigen (Cihak, Ziegler & Kölsch, 1981a).

Demonstration of induction of suppressor cells in experiments simulating early phases of tumour growth

In order to mimic the early phases of tumour growth with respect to antigen concentration, groups of BALB/c mice were injected i.p. with exponentially increasing numbers of syngeneic, irradiated ADJ-PC-5 plasmacytoma cells. The initial injections began with two tumour cells/mouse. Subsequent doses were doubled after each injection according to the generation time of the tumour. The injection protocols for the different groups were started on 15 subsequent days, such that at day 17 group I had received about 10^5 cells whereas group VIII had received only eight cells (Fig. 3). When the total protocol was terminated the various groups of mice represented different stages of tumour growth with respect to antigenic load.

Seven days later PEC and SC from mice of the groups representing the various stages of simulated tumour growth were tested for their possibly suppressing effect on induction of a primary T-cell cytotoxic response of BALB/c SC against ADJ-PC-5 plasmacytoma cells (Fig. 4). PEC from groups I–III showed a complete suppression, those from group IV a partial suppression of induction of T-cell cytotoxicity. PEC from group V–VIII had no suppressive activity (groups VII and VIII not shown). PEC from normal, untreated animals (control in Fig. 4), as well as those from BALB/c mice which had received EL4 cells according to protocols for experimental groups I–VIII did not suppress the induction of ADJ-PC-5 specific T cells (group I shown by open triangles in Fig. 4). SC were in no case suppressive. Thus at early stages the suppressor cells seem to be localized in the peritoneal cavity e.g. the site of the tumour.

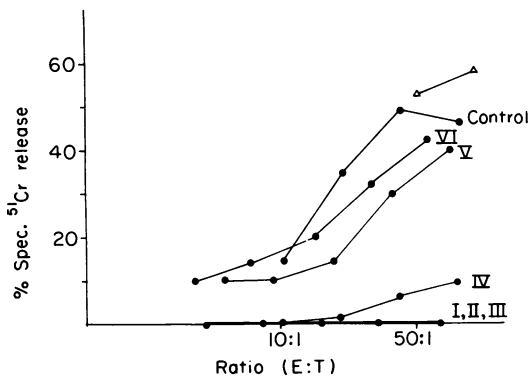

Figure 4. Test for suppression of primary *in vitro* T-cell cytotoxicity of BALB/c SC against ADJ-PC-5 plasmacytoma cells by PEC from animals which have accumulated different doses of irradiated ADJ-PC-5 cells. PEC from mice of group I, II and III inhibit completely and those from group IV inhibit partially the induction of a primary T-cell cytotoxicity. PEC from mice treated with less than 10^3 ADJ-PC-5 cells, or with up to 10^5 EL4 cells according to protocol in Fig. 3 (△) as well as PEC from untreated mice (control) exerted no suppression.

Table 1. Lack of T-cell cytotoxicity of PEC from BALB/c mice treated according to protocol of Fig. 3

Group	^{51}Cr release (%) from labelled ADJ-PC-5 target cells	
	Day 0*	Day 8†
II	0	0
IV	0	0
VI	0	0
VIII	0	0
Control‡	0	32

* PEC were tested immediately after removal from the peritoneal cavity for cytotoxicity at a ratio 100:1.

† PEC were co-cultivated with ADJ-PC-5 for 8 days and then assayed for cytotoxicity at a ratio 10:1

‡ A primary T-cell cytotoxic response of BALB/c SC against ADJ-PC-5 served as control. Data for groups I, III, V and VII are not shown.

Absence of cytotoxic T cells in mice in which early phases of tumour growth were simulated

PEC from mice treated according to protocol of Fig. 3 were tested for cytotoxic activity. Cytotoxic activity was neither found immediately upon removal from the peritoneal cavity nor after culturing them for 8 days in presence of ADJ-PC-5 cells (Table 1). Thus under the experimental conditions used, induction of suppression is neither preceded nor paralleled by activation of cytotoxic T cells.

Characterization and specificity of the suppressor cells

PEC from BALB/c mice treated with up to 10^5 ADJ-PC-5 cells according to scheme in Fig. 3 were separated into plastic adherent and non-adherent fractions. Both fractions are able to suppress a

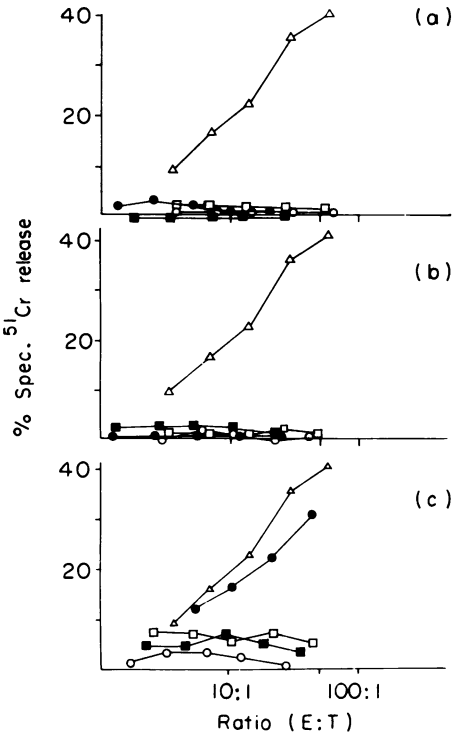

Figure 5. Characterization of PEC suppressor cells. (a) PEC from BALB/c mice which had received 10^5 irradiated ADJ-PC-5 tumour cells according to Fig. 3 (group I) were assayed for suppressive activity with or without depletion of T cells. In the unfractionated population suppression was resistant to treatment with anti-Thy-1.2 plus C. (b) Suppression by plastic adherent PEC from group I mice was also resistant to treatment with anti-Thy-1.2 plus C. (c) Suppression by plastic non-adherent PEC was abrogated after treatment with anti-Thy-1.2 plus C. Control response without PEC (△—△); untreated PEC (○—○); treatment of PEC with anti-Thy-1.2 plus C (●—●); treatment with anti-Thy-1.2 alone (□—□); treatment with C alone (■—■).

primary T-cell cytotoxic response against ADJ-PC-5 plasmacytoma cells (Fig. 5). The activity can be removed from the non-adherent population by treatment with anti-Thy-1.2 antibodies plus complement (Fig. 5c).

The activity of the adherent fraction and of non-fractionated PEC is resistant to this treatment (Fig. 5a and 5b). Thus two cell types seem to be involved, one being a T cell the other one probably belonging to the macrophage line. Both cells can be removed by passage of PEC over a nylon wool column (Fig. 6). Specificity of suppression was tested in a primary

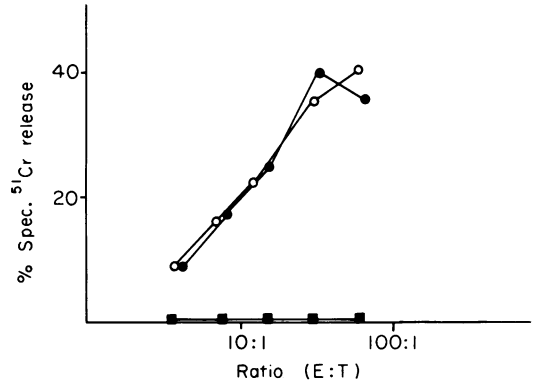

Figure 6. Elimination of suppressor cells from PEC by passage over a nylon wool column. PEC (2×10^7) from group I mice were passed over a nylon wool column and 2×10^6 were recovered and tested for suppressive activity (●—●); compared is the suppression by 2×10^6 unfractionated PEC (■—■). Control in absence of PEC is also given (○—○).

BALB/c anti-ADJ-PC-5 and a BALB/c anti-C57Bl/6 cell cytotoxicity assay (Fig. 7). The allogeneic T-cell response was unaffected by the non-adherent PEC. Thus the T-cell-mediated suppression is specific. Adherent PEC partly suppress the allogeneic response, showing that macrophage activity is to some extent Ts cell dependent. Unfractionated PEC suppress the allogeneic response completely, suggesting an ongoing synergistic action of activated antigen-specific Ts cells and activated macrophages.

DISCUSSION

The *in vivo* immunogenic properties of ADJ-PC-5 plasmacytoma cells in BALB/c mice have been extensively studied (Cihak *et al.*, 1981a, 1981b). Pertinent for the present discussion is that several injections of 10^7 irradiated ADJ-PC-5 cells into BALB/c mice induce a protective immunity against an otherwise lethal challenge with 10^3 living tumour cells. At least part of this *in vivo* immunity is due to T-cell-mediated cellular cytotoxicity . Induction of protective immunity can be influenced by pretreatment of mice with small inocula of irradiated tumour cells. A tumour cell number 10^3 times lower than the one inducing protective immunity reduces the effect of the latter and leads to an enhanced tumour incidence.

The previous demonstration of suppressor cells associated with this enhanced *in vivo* tumour growth

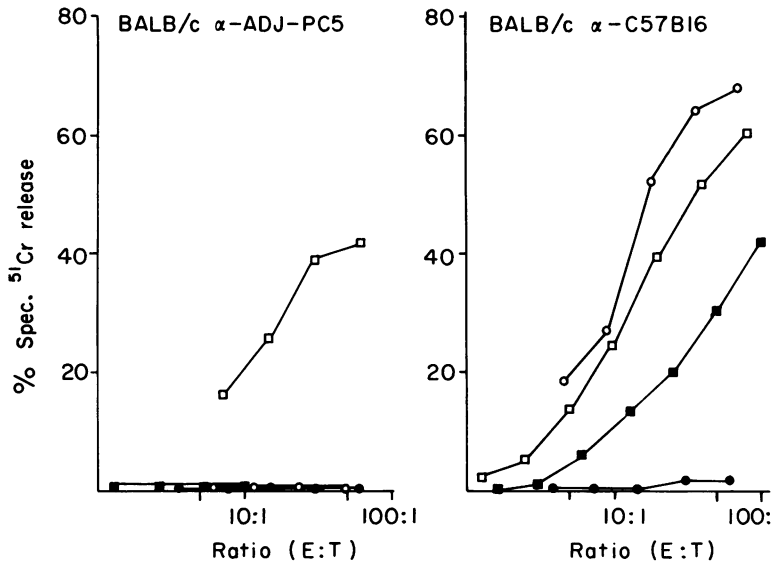

Figure 7. Specificity of suppression of PEC from group I mice. Adherent (■—■) and non-adherent (○—○) PEC were tested for suppression of a primary BALB/c T-cell cytotoxic response against syngeneic ADJ-PC-5 and allogeneic C57Bl/6 SC and compared with the respective control response in absence of PEC (□—□). Suppression by unfractionated PEC is also given (●—●). The non-adherent fraction (2×10^6 cells) suppress specifically only the anti-ADJ-PC-5 response. The adherent PEC fraction (2×10^6 cells) can suppress completely the BALB/c anti-ADJ-PC-5 and partly the BALB/c anti-C57Bl/6 response. Non-separated PEC suppress both types of responses.

(Cihak *et al.*, 1981b) was the basis of the present experimental design. The intention was to simulate very early phases of tumour growth at least with respect to accumulation of antigenic load and to study the hosts immune reactivity under such controlled conditions. This means that the antigen concentration had to increase exponentially according to doubling time of the tumour. By employing several groups of animals and arresting their treatment upon accumulation of different quantities of tumour cells the process could be dissected and studied at any stage. The results show that accumulation of 10^3 or more tumour cells leads to activation of T suppressor cells capable of suppressing specifically a primary *in vitro* T-cell cytotoxic response against the same tumour. In addition a second, plastic adherent cell type, probably a macrophage, is induced and exerts a non-specific suppression. The suppressor T cells seem to be induced before a cytotoxic T-cell response can be activated, since we have never found CTL or CTL precursors in PEC of groups I–VIII animals.

Although T suppressor cells have recently been demonstrated in various tumour models (Fujimoto *et al.*, 1976a, Fujimoto, Greene & Sehon, 1976b; Takei, Levy & Kilburn, 1977; Fujimoto, Matsuzawa, Nakagawa & Tada, 1978; Spellman & Daynes, 1978; Naor, 1979; Behrendt & North, 1980; Perry, Kripke, Benacerraf, Dorf & Greene, 1980; Dye & North, 1981; Tilkin, Schaaf-Lafontaine, van Acker, Boccadoro & Urbain, 1981) little is known about their role in early phases of tumorigenesis. Our present data suggest that in the ADJ-PC-5 system their existence not merely parallels the activation of cytotoxic T cells nor represents a secondary consequence of the growing tumour, but rather is a primary component in the immune reaction towards the neoplastic process promoting tumour growth by undermining in an immunologically specific way a potential anti-tumour reactivity.

It should be pointed out that the ADJ-PC-5 plasmacytoma despite a long transplantation history is still susceptible to lysis by natural killer cells of BALB/c nu/nu mice but no activity is found in BALB/c mice (unpublished results). The lethal dose of this malignant tumour killing 50% of the animals is thirty cells. Thus a NK defence must be unable to cope with such a few cells and seems to play little role in the BALB/c

anti-ADJ-PC-5 response. One tends to believe from the immunization data (Cihak *et al.*, 1981a) that the T-cell immune system could play a major role; but potential elimination of tumour cells by cytotoxic T cells is prevented through early activation of T suppressor cells.

The importance of the findings lies in the fact that the antigen dose inducing suppressor cells is 10^3 times lower than the one which can induce protective immunity *in vivo*. Thus for the ADJ-PC-5 plasmacytoma, a potential T-cell immune response seems to be undermined due to activation of T suppressor cells long before the capacity of inducing protective immunity is reached. It is tempting to speculate that a mechanism similar to the one described here is *in vivo* not only responsible for 'sneaking through' (Old, Boyse, Clarke & Carswell, 1972; Mengersen, Schick & Kölsch, 1975; Gatenby, Basten & Creswick, unpublished) and 'dilution escape' (Bonmassar, Goldin & Cudkowicz, 1971) but might also operate in spontaneous tumorigenesis.

ACKNOWLEDGMENTS

We thank Ms I. Berkenkamp for skilful technical assistance and Mrs H. Storkebaum for help in preparing the manuscript. The work was supported by grant 11 2851 at the Stiftung Volkswagenwerk to E.K.

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