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Elimination of cycling CD4$^+$ suppressor T cells with an anti-mitotic drug releases non-cycling CD8$^+$ T cells to cause regression of an advanced lymphoma

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SUMMARY

This paper describes a model of successful immunotherapy of advanced lymphoma based on the selective elimination of cycling tumour-induced suppressor T cells. It shows that a single injection of the anti-mitotic drug, vinblasting (Vb), results in complete regression of a large L5178Y lymphoma and its metastases, but not if it is growing in an immunocompetent host. Vb-induced, immunologically mediated tumour regression was dependent on the anti-tumour function of CD8$^+$ T cells, because regression was prevented by depleting the host of this subset of T cells 24 hr after Vb was given. Regression was also prevented by infusing the host with Vb-sensitive, CD4$^+$ T cells from a tumour-bearing donor. These and other results are in keeping with the interpretation that Vb-induced regression of the L5178Y lymphoma depends on the ability of the drug to eliminate CD4$^+$ suppressor T cells that are replicating, and to spare non-replicating CD8$^+$ effector cells. It is suggested that at an advanced stage of growth of the L5178Y lymphoma the host possesses an acquired population of antigen-primed CD8$^+$ effector T cells that are unable to become activated in response to abundant tumour antigen because of the dominant influence of CD4$^+$ suppressor cells. Activation of these CD8$^+$ T cells was indicated by the finding that they were rapidly converted from being cyclophosphamide (Cy) resistant to being highly Cy sensitive within 48 hr of giving Vb.

INTRODUCTION

It has been hypothesized (North, 1985) that the immune response evoked by certain immunogenic murine tumours fails to develop sufficiently in magnitude to cause tumour regression because it is down-regulated by suppressor T cells. Evidence in support of this hypothesis is seen in demonstrations (Berendt & North, 1980; North & Bursuker, 1984; North & Dye, 1985) that the premature abridgement of effector T-cell production and loss of already acquired effector T cells by tumour-bearing mice is temporally associated with the production of CD4$^+$ T cells that are convincingly capable of mediating suppression in an adoptive immunization assay. Causal evidence that CD4$^+$ suppressor T cells can suppress the production of effector T cells consists of the demonstration that depleting a tumour-bearing host of suppressor T cells can result in its acquiring enough effector T cells to cause complete regression of a relatively large tumour. This can occur after (i) treating the host with cyclophosphamide (Cy) at the beginning of tumour growth (Awwad & North, 1989a); (ii) exposing it to sublethal γ-radiation early in tumour growth (Awwad & North, 1988b; North, 1986); or (iii) injecting it at a later stage of tumour growth with anti-CD4 monoclonal antibody (mAb) (Awwad & North, 1989b). Obviously the success of this type of therapy depends on the ability of the therapeutic agent to destroy suppressor cells selectively, and this depends on giving the agent at a stage of the anti-tumour immune response when effector T cells are resistant to its action. For this reason Cy must be given at the beginning of the immune response before effector T cells begin to replicate and expand in number (Awwad & North, 1989a), whereas radiation must be given after replication of effector T cells begins (Awwad & North, 1988b; North, 1986). On the other hand, in order for anti-CD4 mAb to be therapeutic, it must be given to a host bearing a tumour that evokes an immune response that is exclusively mediated by CD8$^+$ T cells (Awwad & North, 1989b). In all cases, regression failed to occur if, at the time of treatment, the tumour burden was large enough to have induced dominant suppression.

The purpose of this paper is to show that it is possible to overcome dominant immunosuppression at a late stage of tumour growth and cause immunologically mediated complete regression of an advanced lymphoma by giving the host a single injection of the anti-mitotic drug vinblastine (Vb). The results are consistent with the interpretation that, at a late stage of

Abbreviations: Cy, cyclophosphamide; mAb, monoclonal antibody. TXB, mice made T-cell deficient by thymectomy and irradiation; Vb, vinblastine.

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tumour growth, functionally suppressed, non-cycling $CD8^+$ effector T cells co-exist with a functionally dominant number of cycling $CD4^+$ suppressor T cells, and that Vb is therapeutic by virtue of its ability to destroy the latter cells selectively.

MATERIALS AND METHODS

Mice

Ten- to 12-week-old female B6D2F1 (C57BL/6 × DBA/2) mice, purchased from the Animal Breeding Facility at Trudeau Institute (Saranac Lake, NY), were employed in these studies. These mice are free of common viral pathogens according to the result of routine testing done by Charles River Professional Services, Wilmington, MA.

Tumours

The L5178Y T-cell lymphoma and P815 mastocytoma syngeneic in DBA/2 mice were passaged as ascites, harvested, cryopreserved over liquid nitrogen, and prepared for implantation as described before (Awwad & North, 1989a). The origin of these tumours has also been described in a previous publication (Awwad & North, 1989a). For experiments, 10^6 tumour cells in 0·05 ml of phosphate-buffered saline (PBS) were implanted i.d. in the belly region, and tumour growth or regression monitored by measuring changes against time in the mean of two tumour diameters measured at right angles.

Passive transfer of spleen cells

Spleen cells were obtained by dicing the spleens from mice bearing large tumours into small pieces and gently forcing the pieces through a 60-mesh stainless screen into PBS. The cell suspension was then washed twice in PBS and resuspended in PBS for i.v. infusion. Recipients received 1 organ equivalent (approximately $1·5 \times 10^8$) of spleen cells.

Depleting T-cell subsets

In vitro depletion of T-cell subsets was achieved by incubating spleen cell suspensions ($1–2 \times 10^7$ cells/ml) with either anti-L3T4 mAb produced by hybridoma clone GK1.5 (Dr F. Fitch, University of Chicago, Chicago, IL) or anti-Lyt-2 mAb produced by clone TIB-210 (American Type Culture Collection, Rockville, MD) for 30 min at 4°. The cells were then treated with an equal volume of a 1:10 dilution of rabbit serum as a source of complement for 30 min at 37°, as described previously (North & Bursuker, 1984). T-cell subset depletion was better than 95%, as determined by cytofluorometry with a FACScan cytofluorograph (Becton-Dickinson & Co., Sunnyvale, CA). *In vivo* depletion of $CD8^+$ T cells was achieved by treating mice with a single dose (0·5 mg) of anti-Lyt-2 mAb i.v., as described previously (Awwad & North, 1989b). This procedure resulted in the elimination of >95% of $CD8^+$ T cells. Control mice received an equal quantity of rat IgG (Organon Teknika Crop., Malvern, PA).

T-cell-deficient mice

Mice were made T-cell-deficient by thymectomy at 4 weeks of age, followed 1 week later by whole-body exposure to 900 rads of γ-radiation. Immediately after irradiation, they received 10^7 bone marrow cells from syngeneic donors i.v. The mice were used in experiments after a further 4–6 weeks.

Reagents

Vinblastine sulphate (Vb) was purchased from Eli Lilly and Company, Indianapolis, IN, as a lyophilized powder. It was reconstituted with sterile distilled water and infused i.v. at a dose of 7·5 mg/kg. This dose of Vb should have been capable of destroying cells entering mitosis for a period of about 15 hr (Valeriote & Bruce, 1965). Cyclophosphamide (Cy) was purchased from Mead Johnson, Evansville, IN. It was dissolved in sterile distilled water and infused i.v. at a dose of 150 mg/kg.

RESULTS

Therapeutic effects of Vb given at different stages of tumour growth

Figures 1 and 2 show the results of an experiment that measured the effect on tumour growth and on host survival, respectively,

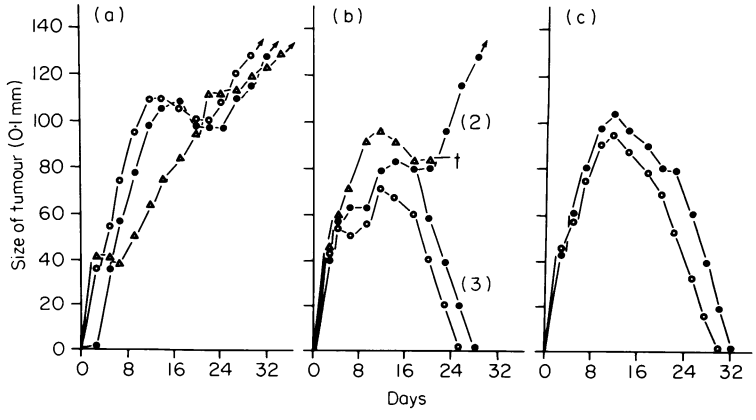

Figure 1. The ability of a single 7·5 mg/kg dose of Vb to cause regression of the L5178Y lymphoma depended on the time of tumour growth it was given. Vb given during the first 3 days (○, control; ●, Day 0; △, Day 3) of tumour growth failed to cause tumour regression (a), whereas Vb caused complete regression if given on Day 4 (○) or 6 (●) (b) or on Day 13 (○) or 15 (●) (c). However, these periods of susceptibility to Vb therapy were separated by a period (Day 10, △) when Vb caused a dramatic increase in the rate of tumour growth (c). Means of five mice per group.

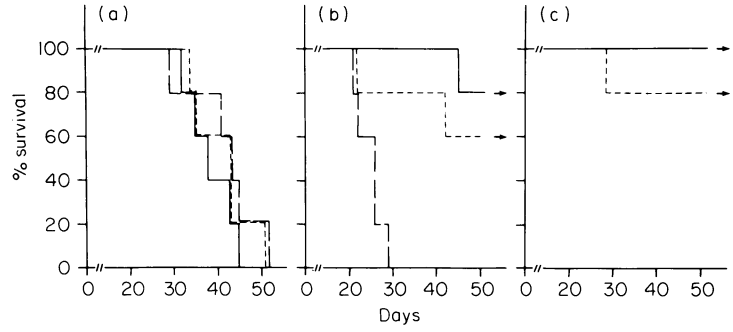

Figure 2. Survival data from the experiment described in Fig. 1 showing that Vb given during the first 3 days (——, control; — —, Day 0; - - -, Day 3) of tumour growth had no effect on survival time (a), in contrast to Vb given on Day 4 (——) or 6 (- - -) (b), or on Day 13 (— —) or 15 (- - -) (c), which resulted in a significant extension of survival time of all mice and apparent cures of most, in that these mice were alive when the experiment was terminated on Day 90. In keeping with the tumour-enhancing effect of Vb given on Day 10 of tumour growth (as shown in Fig. 1) Vb given on this day (— —) caused a significant decrease in host survival time. The results were obtained with five mice per group, and survival measurements were terminated on Day 90.

of a 7·5 mg/kg dose of Vb given on Days 0, 3, 4, 6, 10, 13 or 15 of tumour growth. It can be seen that Vb had little effect on tumour growth and host survival when given during the first 3 days after tumour implantation. It caused regression of the tumour in some mice and increased their time of survival when given on Day 4 or 6 of tumour growth. However, it was most therapeutic when given on Day 13 or Day 15 of tumour growth, when the tumour was relatively large. In these cases the tumour underwent complete regression in all but one mouse and this resulted in long-term (the experiment was terminated at Day 90) host survival. Paradoxically, Vb was highly tumour promotive when given on Day 10 of tumour growth. In this case partial regression was followed by a resumption of rapid growth and in much earlier time to death. It needs to be pointed out in relation to the foregoing results that the L5178Y lymphoma disseminates to the liver at an earlier stage of growth.

Inability of Vb to cause tumour regression in immunoincompetent mice

It is apparent from the foregoing results that the therapeutic action of Vb is not determined soley by its direct toxicity for tumour cells. This is in keeping with the results of an additional experiment that tested the ability of Vb to cause regression of the tumour growing in TXB mice that were incapable of generating anti-tumour immunity. Figure 3 shows that a single dose of Vb had a very limited therapeutic effect against the L5178Y lymphoma growing in TXB mice, regardless of the stage of tumour growth that it was given. Presumably, the small degree of tumour regression that occurred in each case represents the extent of the drug's direct anti-tumour action.

Vb-induced regression of an advanced lymphoma is mediated by Lyt-2$^+$ T cells

It is known from a previous study (Awwad & North, 1989b) that immunity to the L5178Y lympoma is mediated exclusively by CD8$^+$ T cells. It was anticipated, therefore, that Vb would fail to cause regression of a 15-day L5178Y lymphoma if the host were depleted of CD8$^+$ T cells shortly after giving Vb. As can be seen in Fig. 4, a single 0·5 mg dose of anti-Lyt-2 mAb on Day 16 of tumour growth completely prevented tumour regression from

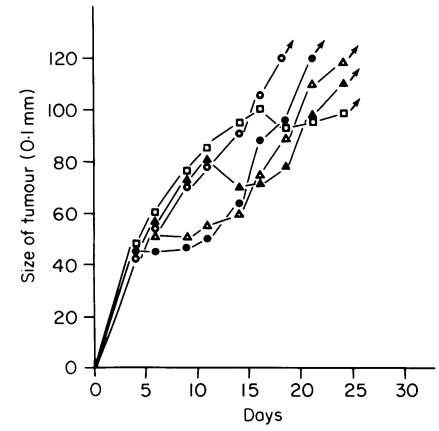

Figure 3. Evidence that a 7·5 mg/kg dose of Vb failed to cause regression of the L5178Y lymphoma growing in TXB mice, regardless of the days of tumour growth (○, control; ●, Day 4; △, Day 6; ▲, Day 10; □, Day 15) that the drug was given. Means of five mice per group.

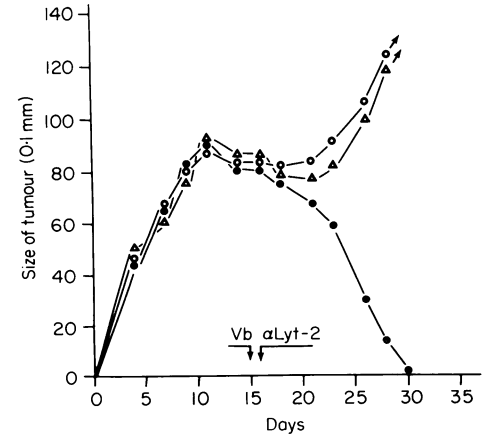

Figure 4. Evidence that Vb-induced tumour regression is mediated by CD8$^+$ T cells. Intravenous infusion of 0·5 mg αLyt-2 mAb (△) on Day 16 of tumour growth completely prevented tumour regression from occurring in response to Vb given on Day15 (Vb αLyt-2). The Vb-treated control mice (●) received 0·5 mg of normal rat IgG. (○) Tumour control. Means of five mice per group.

occurring in response to Vb given 1 day earlier. In this experiment Vb-treated control mice were given 0·5 mg of normal rat IgG. The effect of giving anti-L3T4 mAb has been dealt with previously (Awwad & North, 1989b), where it was shown that this mAb can cause tumour regression in its own right by eliminating CD4+ suppressor T cells.

Vb-induced, CD8+ T-cell-mediated regression appeared to be specific. This is shown by the results presented in Fig. 5, where it can be seen that regression of the L5178Y lymphoma in response to treatment with Vb on Day 15 was not associated with an ability on the part of the host to prevent the progressive growth of an implant of the syngeneic P815 mastocytoma given on Day 16. It is realized, however, that this is a limited test for specificity, and that implants of other tumours may fail to grow in mice in the process of causing regression of the L5178Y in response to Vb treatment. This subject is currently under investigation in this laboratory.

Vb-induced immunity is converted from being Cy resistant to Cy sensitive

The foregoing results indicate that Vb treatment rapidly induces a mechanism of CD8+ T-cell-mediated anti-tumour immunity capable of causing regression of a large L5178Y tumour and its metastases. Moreover, because these CD8+ T cells were resistant to Vb, it follows that most of them were not replicating at the time Vb was given. To obtain evidence as to whether these non-cycling CD8+ effector T cells undergo activation and begin cycling before immunity is expressed, the ability of a single injection of Cy, given at the time of giving Vb or 48 hr later, to interfere with Vb-induced tumour regression was determined. Cy was used instead of a second dose of Vb because it is known that the L5178Y lymphoma is completely resistant to the direct cytotoxic action of Cy. This avoided the complexity of trying to distinguish between enhancement of tumour growth caused by Vb-induced immunosuppression and destruction of the tumour caused by the direct cytotoxic action of Vb.

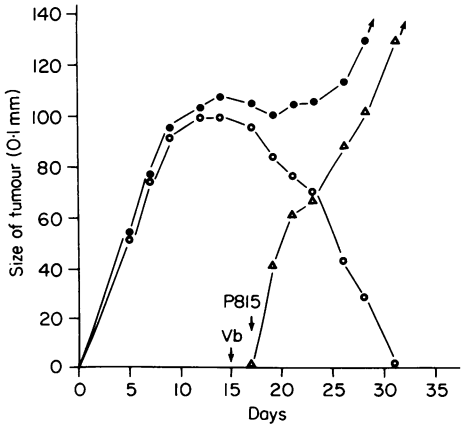

Figure 5. Evidence that immunologically mediated regression of the L5178Y lymphoma is specific. Regression of this tumour in response to Vb (○) treatment on Day 15 was not associated with a capacity of the host to destroy a 2×10^6 implant of syngeneic P815 (△) mastocytoma cells given on Day 16. (●) L5178Y Control. Means of five mice per group.

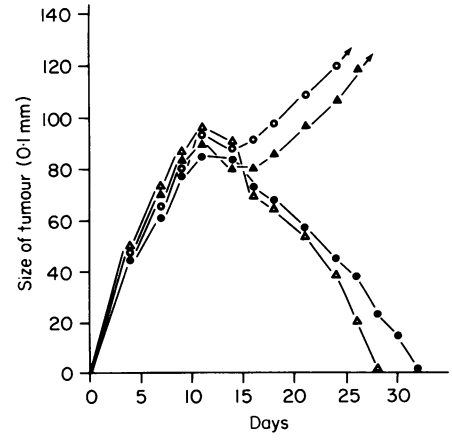

Figure 6. Evidence that Vb-induced regression of a 15-day L5178Y lymphoma (●) depends on the existence of a population of host cells that are highly Cy-resistant at the time Vb is given (△), but which become highly Cy sensitive 2 days later (▲). Cy was given intravenously in a dose of 150 mg/kg. (○) Tumour control. Means of five mice per group.

It was found (Fig. 6) that, whereas a 150 mg/kg dose of Cy given 1 hr after injecting Vb on Day 15 of tumour growth neither aided nor inhibited the ability of Vb to cause tumour regression, the same dose of Cy given 48 hr later completely blocked the therapeutic effect of Vb. This indicates that the CD8+ T cells responsible for Vb-induced tumour regression, as shown in Fig. 4, were converted by Vb treatment from being highly Cy resistant to being highly Cy sensitive.

Inhibition of Vb-induced tumour regression by CD4+ T cells from tumour-bearing donors

It was shown in a previous study (Awwad & North, 1989b), in which anti-CD4 mAb was employed to deplete mice bearing the L5178Y lymphoma of CD4+ T cells, that advanced growth of this tumour evokes the generation of CD4+ T cells that suppress the generation of CD8+ effector T cells. Therefore, it seemed reasonable to interpret the foregoing results as meaning that Vb causes regression of an advanced L5178Y lymphoma by selectively destroying CD4+ suppressor T cells, thereby releasing CD8+ effector T cells from suppression. If so, it should be possible to negate the therapeutic effect of Vb by infusing the host, 24 hr after giving Vb, with CD4+ T cells from a donor bearing a 15-day tumour. It was reasoned further that such suppressor T cells should themselves be Vb sensitive.

As can be seen in Fig. 7, infusing Vb-treated tumour bearers with spleen cells from donors bearing a 15-day tumour completely prevented Vb from causing tumour regression. Moreover, the spleen cells that inhibited tumour regression were CD4+ because they were destroyed by treatment with anti-L3T4 mAb and complement, but not by treatment with anti-Lyt-2 mAb and complement. In addition, they were destroyed by treating the donors with Vb 24 hr before spleen cell harvest.

DISCUSSION

This study shows that Vb-induced regression of the L5178Y lymphoma is an example of successful immunotherapy of an advanced lymphoma based on the selective depletion of sup-

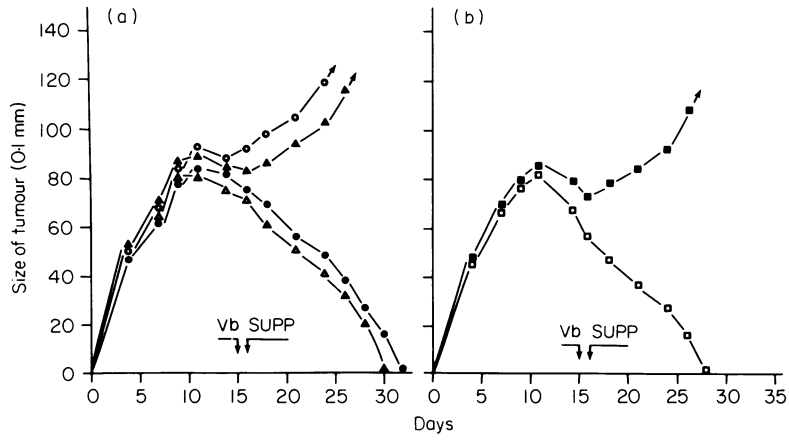

Figure 7. Vb-induced regression of a 15-day L5178Y lymphoma (●) (a) was inhibited by an infusion of suppressor spleen cells from donors bearing a 15-day tumour (▲). The suppressor cells were eliminated from donor spleens by treating the donors 24 hr before killing with Vb (△). The suppressor cells were CD4+ T cells in that they were destroyed by treatment with αL3T4 mAb and complement (□) (b), but not by treatment with αLyt-2 mAb and complement (■). (○) Tumour control. Means of five mice per group.

pressor T cells and the release of immunity from suppression. It was demonstrated that single injection of Vb can result in complete regression of a large tumour and in long-term host survival, but not if the tumour was growing in T-cell-deficient mice. Tumour regression was mediated by CD8+ T cells, because it failed to take place in mice tht were depleted of this subset of T cells with anti-CD8 mAb 24 hr after Vb was given. Regression of the L5178Y lymphoma appeared to be specific to the extent that regression of this tumour was not associated with a capacity on the part of the host to inhibit the progressive growth of an implant of cells of a different immunogenic tumour. These findings agree with already published results (Awwad & North, 1989b) showing that immunity to the L5178Y lymphoma is mediated exclusively by CD8+ T cells. They are also in keeping with recent findings of Mokyr *et al.* (1989), who demonstrated that Melphalan-induced regression of a large progressive MOPC-315 plasmacytoma in BALB/c mice is mediated by CD8+ T cells that are acquired afte the drug is given.

The results presented here indicate tht tumour-specific CD8+ T cells were already present at the time Vb was given on Day 15 of tumour growth, and that they were converted from being Cy resistant to being Cy sensitive within 48 hr. Because Vb and Cy preferentially, though not exclusively, destroy cycling lymphocytes, it follows that a significant fraction of the CD8+ T cells responsible for tumour regression were non-replicating at the time Vb was given, but began to undergo division shortly thereafter. Because it has been shown (Dye & North, 1984) that tumour-sensitized memory T cells, but not functionally active effector T cells, are resistant both to Vb and Cy, it seems reasonable to suggest that a host bearing a 15-day L5178Y lymphoma possesses a state of immunity equivalent to a state of suppressed immunological memory that is carried predominantly by non-cycling CD8+ T cells. It seems reasonable to suggest further that these inactive T cells were derived from activated CD8+ T cells generated as part of a preceding concomitant immune response that is known to develop between Days 6 and 12 of tumour growth in the case of L5178Y lymphoma (Awwad & North, 1989a, b). It is likely that it is because most CD8+ effector T cells are functionally active and replicating on Day 10 of tumour growth that Vb given on this day caused tumour promotion, as shown in Fig. 1.

It is known, in the case of several tumours under study in this laboratory, that concomitant immunity decays after Days 10–12 of tumour growth because of the dominant influence of CD4+ suppressor T cells (Awwad & North, 1988a, b, 1989a, b). It would seem almost certain, therefore, that the therapeutic action of Vb against L5178Y lymphoma depends on the ability of the drug to selectively destroy suppressor T cells, because a significant proportion of them were cycling during the 15-hr period that the drug was active *in vivo* (Valeriote & Bruce, 1965). In this way Vb-resistant, non-cycling CD8+ T cells were released from suppression, and free to become activated in what might be viewed as a secondary anti-tumour immune response. The finding that these suppressor T cells are CD4+ should not be considered unusual, because it has recently been shown that suppressor T cells responsible for induced unresponsiveness to cardiac allografts in rats are exclusively CD4+ T cells (Hall *et al.*, 1989) and belong to a short-lived population (Pearce *et al.*, 1989). It has also been shown that CD4+ suppressor T cells are responsible for down-regulating immunity to leishmaniasis in susceptible mice (Hill, Awwad & North, 1989), and the mounting number of additional publications dealing with CD4+ T-cell-mediated immunosuppression has recently been discussed (Myers, Stuart & Kang, 1989).

Lastly, the demonstration that mice bearing an advanced immunogenic tumour can possess a state of suppressed immunity with a potential to rapidly become activated and cause tumour regression when suppression is eliminated is a clinically relevant example of immunotherapy. The possibility that T-cell-mediated suppression is responsible, at least in part, for the progressive growth of certain human tumours is worth considering, particularly in view of recently published evidence showing that some human tumours are immunogenic enough to evoke the generation of tumour-specific CD8+ cytolytic T cells (Itoh, Platsocus & Balch, 1988; Kawakami, Rosenberg & Lotze, 1988)

and are capable of inducing CD4$^+$ suppressor T cells (Mukherji *et al.*, 1989).

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