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Inhibition of virus replication does not alter malignant rabbit fibroma virus-induced immunosuppression

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SUMMARY

Malignant rabbit fibroma virus (MV) directly suppresses generation of antibody responses and mitogen induced T and B lymphocyte proliferation. We investigated whether this phenomenon required expression of the complete viral genome. Phosphonoacetic acid (PAA) inhibits poxvirus specific DNA polymerases. Adding PAA to cultures reduces both MV replication and mitogen-driven rabbit lymphocyte proliferation in a dose-dependent fashion. A dose of PAA adequate to inhibit MV replication by about 97%, but insufficient to reduce lymphocyte proliferation appreciably, does not affect the ability of MV to suppress lymphocyte proliferation or initiation of antibody production. Spleen cells from MV tumour-bearing rabbits contain very little virus, but inhibit the proliferative and antibody forming responses of normal spleen cells. This activity is shown here to reflect the production by T lymphocytes of a soluble mediator of >25 kD molecular weight. Adding PAA to these mixed spleen cell cultures does not alter the ability of MV to induce T suppressor activity in host lymphocytes. Thus, these immunosuppressive capabilities of MV appear to reflect early MV gene functions.

Keywords malignant rabbit fibroma virus immunosuppression phosphonoacetic acid

INTRODUCTION

Malignant rabbit fibrimoma (MV) was isolated by us and found to be essentially a recombinant between Shope fibrimoma virus (SFV) and rabbit myxoma virus (MYX) (Strayer *et al.*, 1983a; Block, Upton & McFadden, 1985). MV induces a unique clinical syndrome characterized by disseminated fibroxanthosarcoma, severe immunologic impairment, and death from Gram-negative infection (Strayer *et al.*, 1983a, b; Corbeil *et al.*, 1983; Skaletsky *et al.*, 1984). Virus antigen is detectable throughout the reticulendothelial system (Strayer & Sell, 1983), and infectious virus can be recovered from almost all lymphoid organs. As few as 20 infectious units of MV administered intradermally are uniformly lethal.

During MV-induced oncogenesis, lymphocytes from tumour-bearing rabbits do not respond to the optimal B and T lymphocyte mitogens, which are, for rabbits, anti-Ig serum and Con A respectively (Strayer *et al.*, 1983a). These animals do not initiate immune responses to antigens administered following virus infection, though antibody responses initiated before MV inoculation persist (Strayer *et al.*, 1983c). *In vitro*, MV directly inhibits both lectin-induced mitogenesis and the initiation of an antibody response but, analogous to the in-vivo situation, does not abrogate

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antibody production in progress. Spleen cells and virus-free spleen cell culture lysates from MV tumour-bearing rabbits, on the other hand, slightly decrease Con A-induced proliferation but suppress antigen-induced proliferation as well as ongoing antibody responses (Strayer *et al.*, 1983c). Thus, the characteristics of direct virus-induced immunosuppression differ somewhat from those of immunosuppression by spleen cells derived from tumour-bearing rabbits.

We report here our studies to determine at what point during MV replication the genes responsible for virus-induced immunosuppression, both direct and indirect, are expressed. For simplicity, we refer to the 'gene' and 'site' in the MV genome responsible for the observed effects, understanding that more than one may be involved. We also discuss the nature of the host cell population responsible for the observed suppressor activity. Phosphonoacetic acid (PAA), an inhibitor of poxvirus DNA polymerases (Overby, Duff & Mao, 1977), was used to restrict viral replication to early events in infected lymphocytes for these studies.

MATERIALS AND METHODS

Animals. New Zealand white female rabbits were obtained from local suppliers. They were killed by intravenous injection of Euthanol or Ketamine.

Virus. The details of MV isolation and characterization are given elsewhere (Strayer *et al.*, 1983a, b). Briefly, MV is grown and titred using RK-13 cells, according to the procedures of Verna & Eylar (1962) and Padgett, Moore & Walker (1962) for SFV. SFV, Patuxent strain, was the kind gift of Dr W. A. F. Tompkins of the University of Illinois, Urbana, Illinois. Both viruses (SFV and MV) were purified by two cycles of plaque-to-plaque purification, grown to working titres, and stored at −70°C (Strayer *et al.*, 1983b).

Lymphocyte culture procedures. Modified Mishell-Dutton cultures were used as described elsewhere (Redelman *et al.*, 1976). Sheep erythrocyte-primed rabbit spleen cells were immunized in 96-well microtitre plates (Falcon Plastics) in supplemented RPMI-1640 (GIBCO) at 10^5 to $2{\cdot}5 \times 10^5$ cells/0·2 ml in 5% CO for 3 to 5 days and assayed for direct plaque forming cells (pfc) using a modified Jerne-Nordin plaque assay (Jerne & Nordin, 1963). Results (pfc) are reported as mean pfc ± s.e.m. on the day of maximal response. Proliferation assays were performed in the same fashion. Concanavalin A (Con A, Miles-Yeda, Rehovot, Israel) was used at 5 μg/ml final concentration (1 μg/200 μl culture). The ammonium sulphate precipitable fraction of goat anti-rabbit Ig serum was titred for optimal response and used similarly. Both mitogens were added at the time of culture initiation. Responses were measured 24 h after a 0·1 μCi of ^{125}IUdR (New England Nuclear). Cultures were pulsed on days 2, 3, and 4. Results are reported as the geometric mean of ct/min ^{125}IUdR incorporation on the day of maximum responsiveness.

Spleen cell populations. Goat anti-rabbit T cell serum (ATS) was used (the kind gift of Dr Stewart Sell). Its properties are described by Redelman *et al.* (1976). Its cytotoxic activity is restricted to T lymphocytes.

Macrophages were prepared from ATS-treated spleen cells by plastic adherence followed by gentle washing of non-adherent cells from the tissue culture plate.

T cells, where prepared, are enriched from normal spleen cells by removing B and adherent cells using plastic plates coated with goat anti-rabbit Ig serum (Redelman *et al.*, 1976). T lymphocytes were removed following 1·5 h incubation at 4°C. This procedure, in our hands, removes B lymphocytes effectively (D.S. Strayer *et al.*, unpublished observations).

Inhibitors. PAA (Sigma Chemical Co.) was dissolved in RPMI-1640 and added to cultures at the desired concentration.

RESULTS

MV induced suppressor activity. Before determining the effects of PAA on the ability of MV to induce host suppressor activity, the source of MV-induced host suppressor function was determined. This was done in two ways. Spleen cell subpopulations from MV tumour-bearing

Table 1. Cell population responsible for the generation of soluble suppressor activity in MV tumour-bearing rabbits

Supernatant fraction from	Cell source	Population	pfc/10^6*	Proliferation†
None	—	—	1430	12,303 (4·09)
Normal	Spleen	—	2630	26,303 (4·42)
Normal	Spleen	Plastic adherent	2730	15,849 (4·20)
Normal	Spleen	Plastic nonadherent	1600	18,197 (4·26)
Normal	Spleen	ATS-treated	1430	7,244 (3·86)
Normal	Thymus	—	1300	19,055 (4·28)
MV	Spleen	—	100	2,692 (3·43)
MV	Spleen	Plastic adherent	2070	20,417 (4·31)
MV	Spleen	Plastic nonadherent	100	4,571 (3·66)
MV	Spleen	ATS-treated	1860	25,547 (4·39)
MV	Spleen	Anti-Ig nonadherent	0	2,291 (3·36)
MV	Thymus	—	3030	22,387 (4·35)

Normal or MV tumour-bearing rabbit spleen cells were prepared and treated as indicated to obtain populations enriched for adherent or T cells, or depleted of adherent or T cells. These cell populations were cultured for 2 days at 5×10^6/ml. The resulting supernatant fractions were u.v.-treated and added to normal SRBC-immunized spleen cell cultures. Antibody and proliferative responses were measured on days 3 through 5 and are shown here on the day of highest control responsiveness. These results are for a typical experiment.

* Plaque forming cells/10^6 cultures SRBC-primed spleen cells.

† Incorporation of ^{3}H-thymidine, calculated as geometric mean ($\log_{10}$ (ct/min ^{3}H-thymidine)) of replicate samples. Figure shown is antilog$_{10}$ of geometric mean. The logarithmic form is shown in parenthesis.

rabbits were used to generate soluble suppressor activity *in vitro*. T lymphocyte-depleted spleen cells were prepared by ATS treatment. Adherent cells were prepared from ATS treated spleen cells. T lymphocyte-enriched spleen cells were prepared by removing those spleen cells that adhere to plastic plates coated with goat-anti-rabbit Ig. These cells were prepared from normal rabbits and animals which had received MV 7 days previously. After 2 days of incubation, supernatant fractions were treated with U.V. light to remove infectious MV. These supernatant fractions were then added to SRBC-immunized spleen cell cultures from SRBC-primed rabbits.

Results (Table 1) indicate that MV-induced host suppressor activity is not observed in supernatant fractions from ATS-treated spleen cells. Adherent cells (macrophages) do not produce detectable suppressor activity either. Thus, T lymphocytes appear to be responsible for producing MV-induced suppressor activity.

In addition, spleen or thymus cells from normal or MV tumor bearing rabbits were added to cultures of SRBC-primed spleen cells. Fractionation of spleen cells was performed as described above. Table 2 shows that fractionated spleen cells from normal rabbits have minimal effects on sheep erythrocyte responses, both antibody-forming and proliferative. Comparable studies with populations with MV tumor-bearing rabbits, when added to normal SRBC cultures, indicate that T lymphocytes show suppressive activity, rather than plastic adherent or B cells. In both cases (Tables 1 and 2) there is no evidence that thymus cells are capable of elaborating suppressor activity.

To determine the approximate size of the suppressor factor(s) secreted by these T cells, supernatant fractions containing suppressor activity were dialysed through membranes of about 25 K molecular exclusion size. These supernatant fractions, as well as controls, were added

Table 2. Cell populations responsible for MV-induced suppressor function in tumour-bearing rabbits

Cells from	Source	Treatment	pfc/10^5*	Proliferation
None	—	—	1180	69,183 (4·84)†
Normal	Spleen	—	490	83,176 (4·92)
Normal	Spleen	Plastic adherent, ATS	1060	61,660 (4·79)
Normal	Spleen	ATS	323	37,154 (4·57)
Normal	Thymus	—	867	74,131 (4·87)
MV	Spleen	—	3	4,075 (3·61)
MV	Spleen	Plastic adherent, ATS	1120	85,114 (4·93)
MV	Spleen	ATS	1013	48,978 (4·69)
MV	Thymus	—	723	66,069 (4·82)

Spleen and thymus cells from normal and MV tumour-bearing rabbits were prepared. They received the fractionation treatments indicated to remove T cells or enrich for macrophages, and were added to SRBC-immunized cultures of SRBC-primed spleen cells. Antibody and proliferative responses were measured on days 3 to 5 and are shown here for a representative experiment on the day of maximal control responsiveness.

* Direct plaque forming cells/10^5 SRBC-primed spleen cells.

† Incorporation of ^{3}H-thymidine as calculated by the geometric mean ($\log_{10}$ (ct/min ^{3}H-thymidine)) of replicate samples. The geometric mean ($\log_{10}$) is shown in parentheses. The antilog$_{10}$ is also shown.

comparably to SRBC-immunized cultures from sheep erythrocyte-primed rabbits. We found that suppressor activity was retained by dialysis membranes of 25 K pore size: normal pfc/10^5 spleen cells, 493; +day 7 supernatant fractions 33; +7 day supernatants dialysed using 25 K pore size membrane, 33. Similarly, suppression of proliferative responses by day 7 MV spleen cell supernatant fractions was not affected by removal of soluble materials of less than 25,000 daltons (data not shown). Thus, the suppressor molecule(s) generated by day 7 MV spleen cells is $>25{,}000$ molecular weight.

Effects of PAA on virus proliferation. Phosphonoacetic acid was added in increasing doses at the time of culture initiation to spleen cells from tumour-bearing rabbits. The cultures were harvested at different time intervals and the amount of MV present was determined by plaque assay. Figure 1 shows that PAA inhibits MV growth in a dose-dependent manner. Maximal inhibition was observed at concentrations of the drug in the 100 μg/ml to 200 μg/ml range. At these dosages, MV titres reached by such spleen cells do not exceed 1 to 3% of the levels reached by non-PAA exposed spleen cell cultures. Thus, while complete inhibition of MV replication is not achieved, considerable reduction (1·5 to 2 $\log_{10}$) is obtained.

Effects of PAA on lymphocyte proliferation. The direct effects of PAA on lymphocyte responsiveness was assayed. PAA was added at the time of culture initiation to cultures of rabbit spleen cells. These were harvested on days 3 to 5 following 24 h exposure to ^{125}IUdR. PAA does not inhibit lymphocyte responsiveness to B or T lymphocyte mitogens appreciably at concentrations of 100 μg/ml or less (Fig. 2). At 200 μg/ml, we observed about 40% reduction in ^{125}IUdR incorporation in response to anti-Ig and Con A. 'Background' lymphocyte proliferation was not affected.

MV replication is thus inhibited by 95–99% in the presence of 100–200 μg/ml PAA. These concentrations of PAA have little direct effect on the lymphocyte function measured. To determine whether inhibition of late gene functions by PAA (Overby *et al.*, 1977) inhibits direct effects of MV on lymphocytes of MV-induced T suppressor lymphocyte activity, we must also show that the small residual amount of infectious MV produced in PAA cultures is not of itself capable of inhibiting

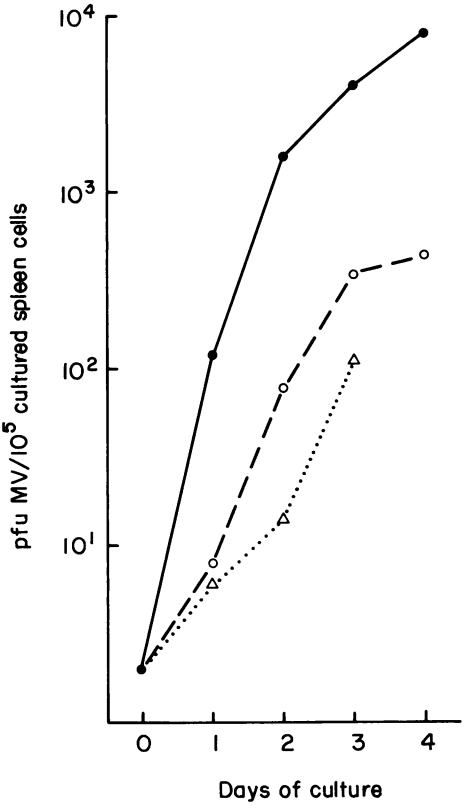


Fig. 1. Effects of PAA on MV replication PAA was added at different concentrations to cultures of spleen cells from MV infected rabbits. Following harvesting, MV recovered from these cultures was titred. No PAA (●); PAA 100 μg/ml (○); PAA 200 μg/ml (△).

lymphocyte function directly without the induction of T suppressor activity. This demonstration is attempted in the following section, where we try to show first that the direct effects of MV on lymphocyte function are not inhibited by preventing MV replication and thus late gene expression; second that MV-induced host-derived suppressor activity is not dependent on late gene expression; and finally that the immunosuppression observed in these two situations cannot either, in magnitude or kinetics, be explained by the effects of the small residual amount of MV capable of expressing its complete genome.

Effects of different amounts of MV on T cell proliferation. We next sought to determine the amount of MV necessary to inhibit B and T lymphocyte functions, relative to the inhibitory effects of PAA on MV replication. Thus, 10^5 spleen cells from rabbits bearing MV-induced tumours will produce 100 plaque forming units (pfu) MV by day 2 of culture and 800 by day 3–4 in the presence of 100 μg/ml PAA. With 200 μg/ml PAA, 100 pfu MV are produced by day 3. Our experience with large doses of MV (10^4 pfu/culture) is that such doses inhibit B and T cell proliferation after a 2 day lag period (Strayer *et al.*, 1983c). Thus, to determine whether the small number of infectious units of MV produced over the time course of our lymphocyte cultures was adequate by itself to inhibit lymphocyte function, we examined the time course of MV-induced inhibition of T and B lymphocyte functions. T cell responses were examined first. We therefore added 10^4 pfu MV or 10^3 pfu MV (multiplicity of infections (MOI) = 0·1, 0·01 respectively) at time of culture initiation to spleen cell cultures stimulated with Con A. Proliferation was measured at 24 h ^{125}IUdR incorporation thereafter. Figure 3 shows that 10^4 pfu causes considerable inhibition of Con A-

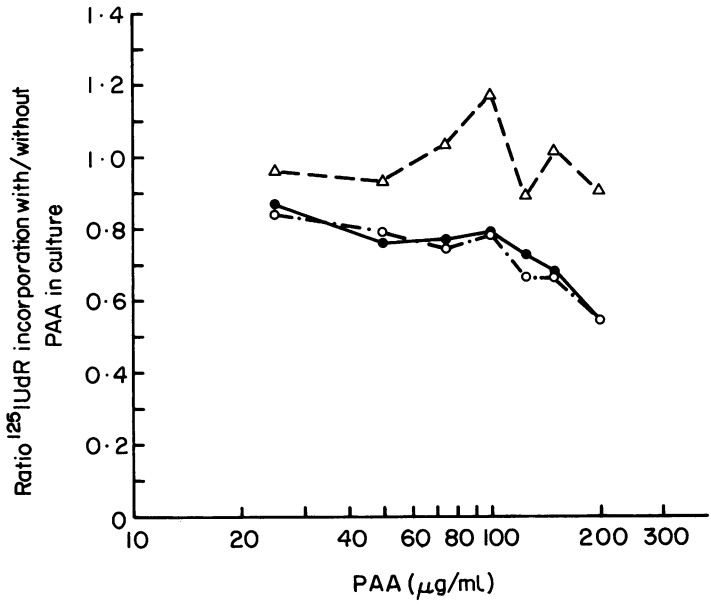


Fig. 2. Effects of PAA on anti-Ig- and con A-induced spleen cell proliferation. Rabbit spleen cells were cultured in modified Mishell-Dutton cultures with anti-Ig serum or 5 μg/ml Con A. On days 2 to 4 0·1 μCi^{125} IUdR was added to each well of these cultures. Twenty-four hours after pulsing, the cultures were harvested. Results shown reflect the maximum ^{125}IUdR incorporation for each group over the course of the several experiments. Effects of PAA on ^{125}IUdR incorporation are shown as the ratio of ct/min IUdR of spleen cells with mitogen + PAA: ct/min (^{125}IUdR) of spleen cells with mitogen alone. Similarly, effects of PAA on background proliferation activity are shown as the ratio of ct/min (^{125}IUdR) of spleen cells + PAA: ct/min of spleen cells without PAA. Background (△); Con A (●); Anti-Ig (○).

stimulated lymphocyte proliferation first observable by day 3. No detectable effect is produced until at least day 5 by 10^3 pfu MV. Even at that time, only moderate inhibition of Con A-induced proliferation is seen at this dose of MV.

Effects of PAA on MV-induced suppression of T lymphocyte proliferation and antibody responses. We then tested whether PA was capable of altering direct effects of MV on T lymphocyte proliferation and antibody production in response to antigen. Rabbits were immunized on day −7 with SRBC. On day 0, their spleen cells were cultured with SRBC as a mitogen. PAA was added at the time of culture initiation. To these cultures, we also added MV, SFV, or no virus. Viruses were added at a multiplicity of infection of 0·1. PAA was not capable of altering the complete suppression of lymphocyte proliferation caused by MV (Table 3). SFV caused a moderate decrease in lymphocyte proliferation which was also untouched by addition of 100 μg/ml PAA.

To determine whether a comparable effect was obtainable with antibody responses, parallel cultures were assayed for antibody production. Direct pfc were measured on days 3–5. MV suppressed the pfc response to SRBC; this suppression was unaffected by PAA at 100 μg/ml (Table 3). Thus, inhibition of MV proliferation did not appreciably alter the ability of MV to inhibit T cell proliferation, or antibody production, by immune spleen cells.

Kinetics and dose dependence of MV-induced immune suppression of B cell functions. We then addressed the question of how much MV was adequate to induce suppression, and, simultaneously, to what extent adding PAA after culture initiation would alter this effect. Normal spleen cells (2×10^5 cells/culture) were cultured with anti-Ig serum (B lymphocyte mitogen). MV was added at different MOI on day 0. PAA was added to these cultures to a final concentration of 200 μg/ml, on days 0, 1, 2 or 3 of culture. We measured 24-h ^{125}IUdR on incorporation on day 4. The results (Fig.

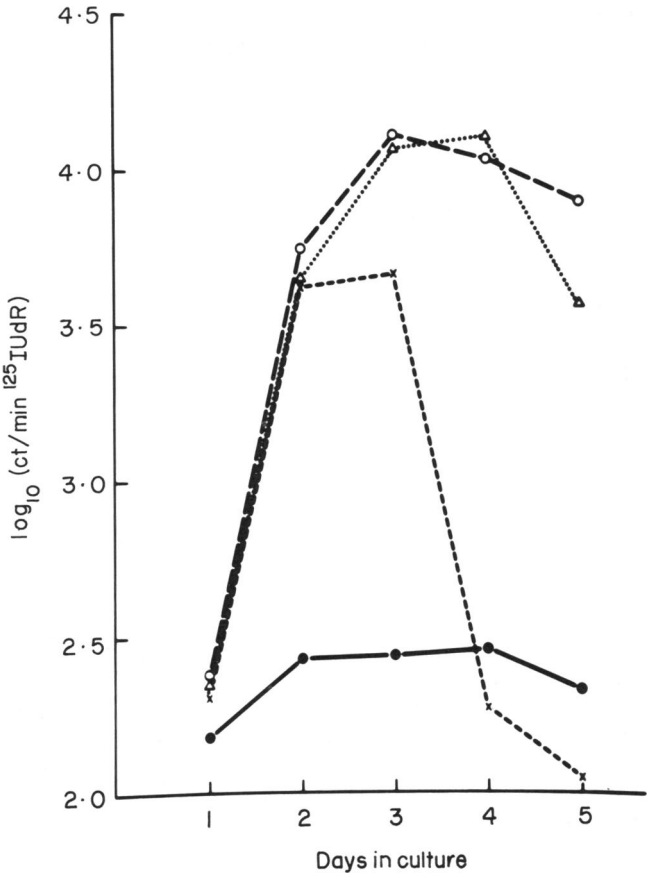


Fig. 3. Time course of the effects of two different doses of MV on Con A-induced lymphocyte proliferation. MV at MOI of 0·1 or 0·01 was added to normal spleen cell cultures on day 0. Proliferation was measured as 24 h ^{125}IUdR incorporation on days 1 to 5. Con A (○); Con A + (MV 0·01) (△) Con A (MV 0·1) (×); bkg (●).

Table 3. Effect of PAA on suppression of immune response by MV

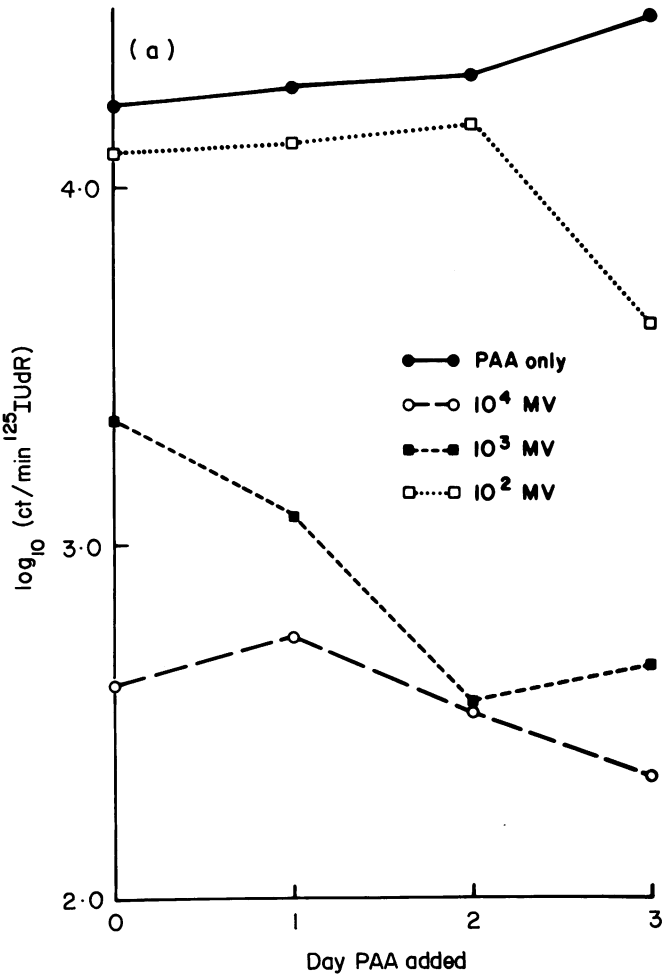
SRBC spleen cells	MV (pfu/well)	SFV (pfu/ well)	PAA (μg/ml)	Responses: ^{125}IUdR uptake (ct/min)	Responses: pfc/10^6 cells
10^5 cells/well	—	—	—	20,684	71,928
10^5 cells/well	—	10^4	—	5,025	6,682
10^5 cells/well	—	10^4	100	4,235	4,848
10^5 cells/well	—	10^4	200	5,664	3,499
10^5 cells/well	10^4	—	—	182	0
10^5 cells/well	10^4	—	100	215	66
10^5 cells/well	10^4	—	200	198	0

Rabbits were immunized with SRBC on day-7. On day 0, their spleen cells were placed in culture with SRBC, with or without MV or SFV at MOI = 0·1, with or without PAA. Proliferation in response to SRBC was measured and is reported here on the day of peak proliferation. Pfc were measured on the day of peak response, and all reported as pfu/10^6 SRBC-immunized spleen cells.

4(a)) indicate that 10^4 pfu MV (MOI = 0·05) was capable of suppressing B lymphocyte proliferation despite inhibition of virus replication from day 0 onwards by PAA. If 10^3 pfu MV (MOI = 0·005) is added, a considerable decrease in proliferation is observed whenever PAA is added. However, inhibition of MV replication on day 0 results in almost one log better responsiveness than if MV replication is not inhibited until day 2 or 3. At the latter time proliferative activity is not appreciably different in cultures receiving 10^3 MV from cultures receiving 10^4 pfu MV. Similarly, adding 10^2 pfu MV to these mitogens results in a significant decrease in B lymphocyte proliferation only if PAA is withheld until 1 day before assay (day 3 of culture).

Where antibody production to SRBC was measured in a similar setting, different results were obtained (Fig. 4(b)). MV at 10^4 or 10^3 pfu/culture (MOI = 0·05 or 0·005 respectively) inhibited SRBC pfc generation regardless of time of PAA addition. If 10^2 pfu were added (MOI = 5×10^{-4}) inhibition of virus replication on day 0 or 1 prevented virus induced suppression of pfc. Inhibition of MV replication later than day 1 of culture did not alter this effect.

Effects of PAA on the suppressive capacity of spleen cells from MV tumour bearing rabbits. Spleen cells from MV tumour-bearing rabbits suppress immune responses of normal spleen cells *in vitro*. Spleen cells from SFV tumour-bearing rabbits either do so only slightly or not at all. These effects are mediated via the cells themselves, and do not reflect the direct effects of virus carried over with the spleen cells from tumour-bearing rabbits (Strayer *et al.*, 1983c). In any event, less than 10 pfu



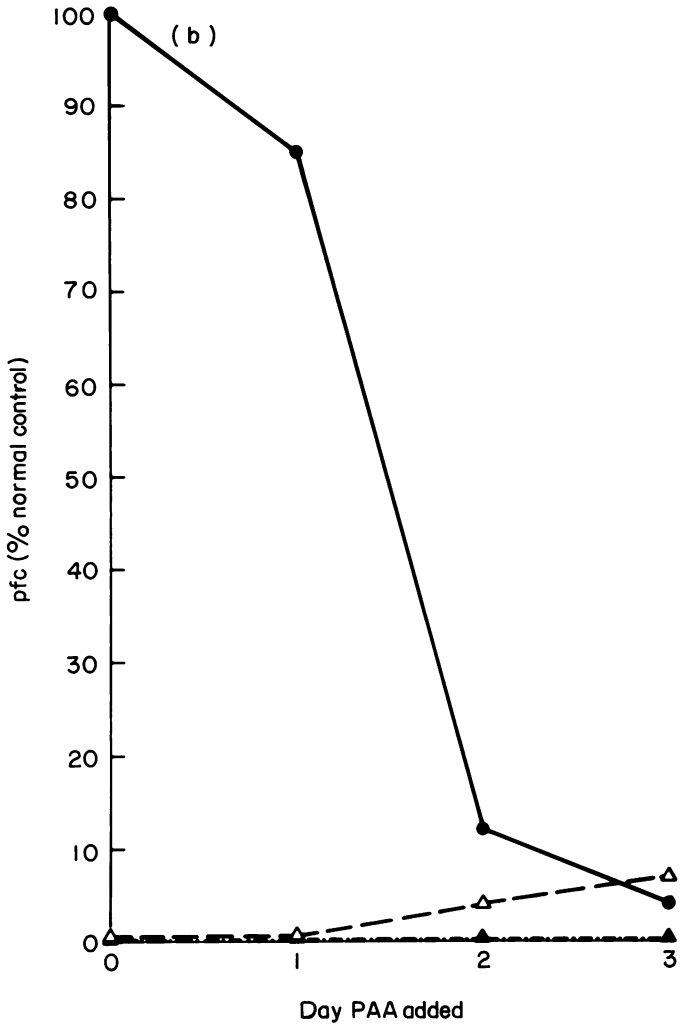


Fig. 4. Effects of MV and PAA on B lymphocyte function. MV was added to cultures containing 2×10^5 SRBC primed or normal spleen cells, at three different doses: 10^4 pfu, 10^3 pfu, or 10^2 pfu. To these cultures, 200 μg/ml PAA was added on day 0, 1, 2 or 3. Proliferation of B cells (a) in response to anti-Ig serum was measured as 24 h incorporation of ^{125}IUdR. Antibody responses (b) are shown as a percentage of the antibody responses of comparable cultures without added virus. (a) PAA only (●); 10^4 MV (○); 10^3 (■); 10^2 MV (□). (b) 10^4 MV (▲); 10^3 MV (△); 10^2 MV (●).

MV are initially detectable in 10^5 spleen cells from tumour-bearing rabbits (D. Strayer *et al.*, unpublished observation). Spleen cells from MV tumour-bearing rabbits do not produce 10^3 MV in the presence of PAA at either 100 μg/ml or 200 μg/ml (Fig. 2). Thus, as maximal amounts of MV are not even available from those cells until day 3 of culture, the amount of whole infectious MV in the PAA-containing cultures is not adequate to suppress directly spleen cell function as assayed.

We therefore sought to determine whether the complete expression of the MV genome was required to induce spleen cells from such rabbits to suppress normal immune responses. Thus, we mixed splenic lymphocytes from rabbits with SFV- and MV-induced tumours with spleen cells from rabbits immunized 7 days previously with SRBC. PAA was added. These cultures were immunized with SRBC as before, and assayed for proliferation and antibody responses on days 3, 4 and 5.

Table 4. Effect of PAA on suppression of immune response caused by MV-exposed spleen cells

SRBC spleen cells	MV spleen cells	SFV spleen cells	PAA (μg/ml)	Responses: IUdR uptake (ct/min)	Responses: pfc/10^6 cells
10^5/well	—	—	—	20,684	71,928
10^5/well	—	5×10^4/well	—	27,447	29,184
10^5/well	—	5×10^4/well	100	24,618	22,268
10^5/well	—	5×10^4/well	200	20,422	14,759
10^5/well	—	2×10^4/well	—	26,458	52,022
10^5/well	—	2×10^4/well	100	18,889	41,099
10^5/well	—	2×10^4/well	200	17,001	14,218
10^5/well	5×10^4/well	—	—	1,482	10,844
10^5/well	5×10^4/well	—	100	2,279	10,346
10^5/well	5×10^4/well	—	200	2,470	1,306
10^5/well	2×10^4/well	—	—	4,101	2,573
10^5/well	2×10^4/well	—	100	7,121	11,649
10^5/well	2×10^4/well	—	200	3,849	5,340

Rabbits were immunized with SRBC on day-7. On day 0, their spleen cells were placed into culture with SRBC, with or without spleen cells from rabbits receiving MV (100 pfu) or SFV (10^7 pfu) on day-7 with or without PAA. Responses were measured as ^{125}IUdR incorporation (ct/min) and pfc/10^6 SRBC-immunized spleen cells.

Spleen cells from MV infected rabbits suppressed normal antibody and proliferative responses. Those from SFV-infected rabbits did so slightly. PAA had no effect on the ability of spleen cells from rabbits with MV-induced tumours to suppress these responses to SRBC on the part of normal cells (Table 4). As shown in Figs 3 and 4, 10^4 pfu MV are required for direct suppression of T cell proliferative responses and 10^3 pfu are required for suppression of antibody responses. These levels are not achieved in the presence of the levels of PAA employed here until day 3–4 of culture, leaving insufficient time for direct virus-mediated suppressive effects, which require about a 2 day lag time (Strayer *et al.*, 1983c). Thus, the suppression observed probably reflects virus-induced but cell-mediated immune suppression. PAA does not alter this affect.

DISCUSSION

Immunological function is impaired by virus infection in clinical, veterinary, and laboratory settings. This phenomenon, first recognized in 1908 (von Pirquet, 1908), has been studied extensively and reviewed several times recently (Woodruff & Woodruff, 1975; Notkins, Mergenhagen & Howard, 1970; Semenov, Zschlesche & Gavrilov, 1977; Black & Hirsch, 1978). The reasons for virus-induced immune dysfunction vary greatly from one virus to the next and from one host to the next.

Only rarely have investigators been able to determine the molecular basis of this type of phenomenon. For retroviruses, low molecular weight glycoproteins seem to be involved. The first discovered of these was in the feline leukaemia virus (FeLV) system. The entire inhibitory effect of FeLV on lymphocyte proliferation was localized to one protein (gp 15) whose action was shown to be inhibition of lectin-induced capping (Mathes *et al.*, 1979; Dunlap *et al.*, 1979).

In an effort to determine the relationship of immunosuppression to the time course of virus gene

expression, we studied the effects of inhibitors of virus replication on MV-induced immune dysfunction. We first studied rifampicin (D. Strayer, unpublished observations), an inhibitor of poxvirus morphogenesis that finds widespread clinical application as an antimycobacterial agent (Hobby, 1969). Rifampicin proved as effective at inhibiting immune responses as it was at inhibiting MV replication. The basis for this effect of rifampicin on lymphocyte mitogenesis is unknown; the drug is not a general inhibitor of mammalian DNA synthesis at the concentrations used.

Phosphonoacetic acid inhibits Herpesvirus DNA polymerase efficiently at concentrations of 100 μg/ml. It does not appear to affect the RNA viruses examined on several other types of DNA virus. PAA is reported to be only moderately inhibitory of vaccinia (Overby *et al.*, 1977). Phosphonoacetic acid proved useful in our hands in inhibiting MV replication, while permitting lymphoid cells to respond to mitogens. PAA at 100 μg/ml dramatically decreases MV replication in lymphocytes without appreciably changing Con A or anti-Ig responsiveness of rabbit spleen cells. While inhibition of malignant fibroma virus proliferation by 100 μg/ml PAA is not complete, a reduction of about 97% (1·5 $\log_{10}$ units) is observed over the course of the 4 day culture period. With 200 μg/ml PAA, a reduction of about 99% (2 $\log_{10}$ units) in MV proliferation occurs. At this higher concentration of PAA, however, mild depression of lymphocyte responsiveness is noted. An even more dramatic decrease in virus growth is observed if RK-13 cells are used rather than lymphocytes as virus targets (D. Strayer, unpublished observations).

We show here that MV-induced host suppressor activity reflects activation of host T lymphocytes to produce suppressor factors of >25 kD molecular weight. ATS treatment, specific for T lymphocytes, abolishes such activity. Adherent cells do not elaborate detectable suppressor activity into their culture supernatant fractions. Thymus cells as well are devoid of such activity, probably owing to their relative immaturity. When lymphocytes from MV tumour-bearing rabbits are examined, T lymphocytes are noted to support replication and express MV antigens in their cell surface most efficiently. Most splenic T cells contain MV antigens. Whether it is these virus-infected T cells or others that mediate the observed T suppressor functions is not yet clear. However, virus-free supernatant fractions from MV-infected spleen cells are capable of inducing suppressor activity in normal spleen cells (D. Strayer, unpublished observations).

B cells also permit virus replication, though less efficiently. Though MV antigens are present systemically throughout the fixed tissue phagocytes (Strayer & Sell, 1983) these cells permit less virus replication than any other spleen cell population (Strayer, Skaletsky & Leibowitz, 1985; Strayer, *et al.*, unpublished observation). Thymus T cells appear not to be infected by MV (Strayer *et al.*, unpublished observation). Additional studies to characterize this T lymphocyte-derived suppressor factor are in progress. However, this activity is quite unstable to freezing and thawing, and deteriorates in storage at −20°C for long periods of time. We have found no evidence for either persistent MV infection or for defective interfering particles as means for effecting immunological dysfunction during MV infection.

We approached the question of PAA inhibition of MV-induced immune dysfunction in two ways. First we sought to examine alterations in the direct effects of MV on lymphocyte function. Second, we tested the ability of PAA to diminish the indirect effects of MV on lymphocyte function: those requiring mediation by lymphocytes from virus-infected hosts.

Inhibition of MV proliferation early in culture is capable of inhibiting the direct suppressive effects of MV on T or B cell proliferation. At low MOI (5×10^{-3}, 5×10^{-4}), some MV proliferation is apparently required for its suppressive effects to be evident. There is most likely a critical concentration of virus necessary for observable immunosuppression, but complete replication of MV DNA does not seem to be a requirement. The direct immunosuppressive capacity of MV thus appears to reflect the functions of early MV gene products, i.e., those encoded in the portions of the genome which do not require DNA replication for their expression (Overby *et al.*, 1977).

We have also addressed the temporal expression of virus genes that are capable of eliciting the production of immunosuppressive factors by host T cells. Spleen cells from rabbits given MV *in vivo* have been shown previously to suppress responsiveness of normal spleen cells when the two populations are mixed in culture. The expression of this suppression does not reflect MV replication Strayer *et al.*, 1983a, c).

In this communication, we report that substantial continued replication of MV in lymphocytes

from MV tumour-bearing rabbits is not needed for the induction of host suppressor T cells. Comparing the time course of the MV replication curve without PAA (Fig. 2) and the lag time required for even 10 pfu MV to alter in-vitro responses (Fig. 3), it appears that the generation of infectious virus and its direct affects on responding lymphocytes are not likely to account for the effects observed when MV-exposed and SRBC-primed spleen cells are mixed even in the absence of PAA.

In conclusion, we have demonstrated that the two observable effects of MV on the immune system, the direct virus effect and the induction of T lymphocyte-derived soluble suppressor factors on the part of the host, are not inhibited by an effective inhibitor of poxvirus DNA polymerase. These observations suggest that both effects are mediated by genes expressed before DNA replication.

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