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Lymphocyte and Macrophage Responses After Vaccinia Virus Infections

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Using a semimicromethod with washed whole blood, in vitro lymphocyte responses of rabbits to intradermal infection with vaccinia virus were studied. Peritoneal exudate macrophages were infected with vaccinia in vitro to determine the time of appearance of activated macrophages. After primary infection, an increase in spontaneous incorporation of thymidine by blood cultures was found as early as 2 days postinfection. This effect was at a maximum at 7 to 10 days, with counts up to 100-fold higher than before infection. Incubation of these cultures with concanavalin A showed a marked decrease in stimulation index as compared with normals. Although only a transient stimulation with vaccinia was found during the acute infection, stimulation indexes of 2 to 3 were obtained during convalescence. Macrophages from rabbits early after infection supported vaccinia replication, whereas those at day 6 or later resisted infection. Macrophage resistance persisted for 2 to 3 weeks. The response of lymphocytes from rabbits reinfected with vaccinia after 15 weeks differed, with a small increase in spontaneous thymidine uptake, a smaller depression in concanavalin A stimulation, and a greater specific response to vaccinia. Macrophage activation occurred earlier and persisted for a longer time after secondary infection.

Previous studies have demonstrated the in vivo production of activated macrophages after bacterial or viral infections (1, 6). Other workers have described the time sequence for the production of sensitized lymphocytes of spleen and peripheral blood after virus infections (3, 13, 14) and, although studies have shown that sensitized lymphocytes stimulated in vitro with the immunizing agent can produce macrophage-activating factors (4, 7, 9, 17), little information is available on the in vivo temporal relationships of macrophage activation and production of sensitized lymphocytes.

The present study describes the time sequence of macrophage and lymphocyte responses after vaccinia infection of rabbits. The in vitro responses of lymphocytes in washed whole blood cultures to virus and to the nonspecific mitogen concanavalin A were determined after primary or secondary infections, whereas peritoneal macrophages from similarly infected rabbits were tested for resistance to in vitro infection with vaccinia virus (18).

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MATERIALS AND METHODS

Cells and culture media. Primary rabbit kidney and chicken embryo fibroblast cultures were prepared by standard methods. Vero cells (a continuous line of monkey kidney cells) and RK13 cells (a continuous line of rabbit kidney cells) were subcultured as required. Cultures were grown in Eagle minimal essential medium (MEM) (GIBCO) supplemented with 0.03% L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% inactivated fetal calf serum (FCS), buffered with 0.075% $NaHCO_3$.

Whole blood cultures were grown in RPMI 1640 medium (GIBCO) supplemented with 10% FCS and glutamine, antibiotics, $NaHCO_3$, and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) as described above.

Viruses. Vaccinia virus, WR strain, was grown in either primary rabbit kidney or Vero cells. The cultures were disrupted by freezing and thawing when they showed almost total cytopathic effect. Cell debris was removed by centrifugation (1,000 × *g* for 10 min), and the supernatant fluid was stored in small aliquots at −70°C. The virus was titrated by plaque formation on Vero cultures with a liquid overlay of maintenance medium.

Semliki Forest virus was obtained from M. A. Chernesky, St. Joseph's Hospital, Hamilton. The virus was grown in chicken embryo fibroblast cultures and titrated in similar cells grown in flat-bottomed microtest plates (Falcon).

Infection of rabbits. Young adult New Zealand

White rabbits, 6 to 8 lb (ca. 27 to 36 kg), were obtained from local breeders. Vaccinia virus (total of 10^7 plaque-forming units [PFU]) was inoculated intradermally into six sites on the shaved back of each animal.

Infection of peritoneal macrophages. Peritoneal macrophages were induced by administration of 50 ml of a sterile 4% suspension of starch. Three days later the anaesthetised animals were exsanguinated by cardiac puncture, and the peritoneal exudate cells were removed by washing with Hanks solution containing 5 U of heparin per ml. The cells were washed twice in Hanks solution and resuspended in MEM (10% FCS) to a concentration of 5×10^5 cells/ml. One-milliliter volumes of cell suspension were distributed into 1-dram (ca. 1-g) glass vials, and the cultures were incubated for 1 h at 37°C in an atmosphere of 5% CO_2 in air. Nonadherent cells were removed by gentle shaking, and the culture medium was poured off. Vaccinia virus diluted in MEM was added (multiplicity of infection, 0.2 PFU/cell) and allowed to adsorb for 1 h at room temperature. Nonadsorbed virus was removed by rinsing three times with MEM, and finally 1 ml of MEM (10% FCS) was added to each culture. Duplicate cultures were frozen at this time and after 48 h of incubation at 37°C. Virus titers were measured after thawing and brief sonication of the cultures.

Lymphocyte cultures. Blood was drawn from the marginal ear vein into heparinized plastic syringes. It was washed three times in Hanks solution (10 ml) and suspended in RPMI 1640 medium to give a 50% suspension. To 1 ml of RPMI 1640 medium, 25 μl of washed-blood suspension and 0.1 ml of antigen or mitogen were added, and the cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air (16). The cells were resuspended by shaking at 24-h intervals.

Triplicate cultures were stimulated with concanavalin A (Calbiochem) either 1 or 5 μg/ml, whereas other cultures were inoculated with two dilutions of vaccinia virus antigen. This antigen was prepared by centrifuging a stock virus pool at 100,000 $\times$ *g* for 1 h and resuspending the pellet in one-tenth of the initial volume. The concentrated virus had a titer of 5.5×10^7 PFU/ml and was inactivated by ultraviolet irradiation. Cultures were inoculated with 0.1 ml of a 1:7 and 1:14 dilution of this preparation. Control cultures were incubated without concanavalin A or virus.

The cultures were incubated for 72 h; 5 h before termination, 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; NET-027 New England Nuclear Corp.) was added. Incubation was terminated by the addition of 5 ml of cold phosphate-buffered saline and cells were pelleted by centrifugation. After freezing and thawing, the pellets were resuspended in 5 ml of phosphate-buffered saline and filtered through glass-fiber filters (Whatman), which were then rinsed with 10 ml of 5% ice-cold trichloroacetic acid followed by 5 ml of methanol. The filters were air dried and placed in scintillation vials containing toluene plus Fluoralloy TLA (Beckman Instruments). Radioactivity was determined in a Beckman LS-230 scintillation counter and expressed as the mean counts per minute of three replicates. Results were expressed as a stimulation index (SI), which is the ratio of counts per minute in concanavalin A- or virus-stimulated cultures to counts per minute in the control cultures from the same animal.

Blood cell counts. Total leukocyte counts were performed on heparinized blood using a Coulter cell counter. Differential counts were performed on May-Gruenwald-Giemsa-stained smears of whole heparinized blood.

RESULTS

Lymphocyte response to a primary vaccinia infection. The responses of typical infected and noninfected rabbits are described. Similar responses were observed in other animals in similar experiments, although the time of maximal response was found to vary by 2 to 3 days.

Control blood cultures from noninfected animals showed a low level of spontaneous thymidine incorporation ranging from 200 to 400 cpm. In contrast, control cultures from infected animals showed a marked increase in incorporation of thymidine, which was evident as early as 2 days postinfection (p.i.) and reached a peak at 8 days, approximately 100-fold over normal cultures (Fig. 1). A second peak in immune cultures was observed at 15 days p.i. and spontaneous counts remained 10-fold greater than normals at 33 days p.i. This secondary peak of spontaneous thymidine incorporation was observed in most but not all of vaccinia-infected rabbits.

Incubation of blood cultures from infected animals with inactivated vaccinia virus showed a pattern of thymidine incorporation similar to unstimulated cultures (Fig. 1); however, when a specific SI was calculated, a twofold transient stimulation of immune lymphocytes was observed on days 7 and 9 (Fig. 2). In convalescence a SI of 8 was observed at day 33. In other animals that were kept for a longer time, SIs of 2 to 3 were recorded up to 15 weeks after infection. As shown in Fig. 2, inactivated vaccinia had a slight toxic effect on normal lymphocyte cultures, resulting in a negative SI.

The response of blood cultures to the nonspecific mitogen concanavalin A was followed throughout the experiments. Cultures from normal animals usually showed an SI of 50 to 100. In contrast, immune lymphocyte cultures showed an apparent marked decrease in response to concanavalin A as early as 2 days after infection. This persisted for 2 weeks and returned to near normal levels late in convalescence (Fig. 3). However, comparison of absolute counts in concanavalin A-stimulated cultures

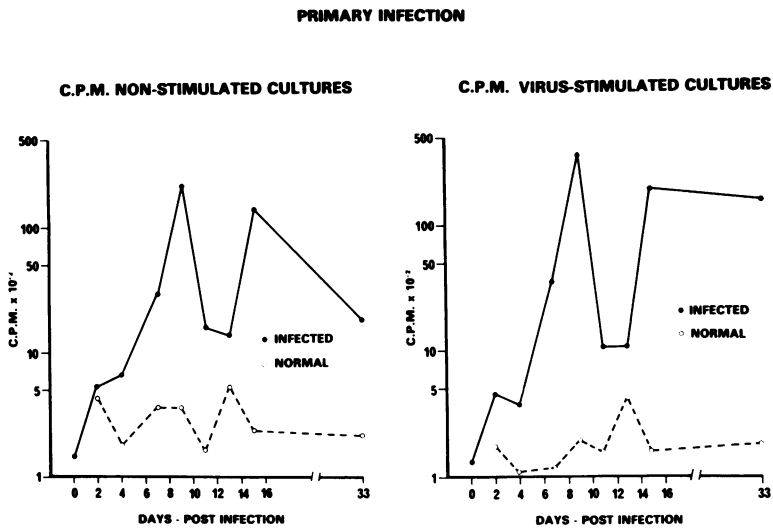

FIG. 1. *[³H]thymidine incorporation of whole blood cultures from control (○) and vaccinia-infected (●) rabbits after a primary intradermal infection with 10^7 PFU of vaccinia virus. The mean of counts per minute is shown for nonstimulated and for vaccinia virus-stimulated cultures.*

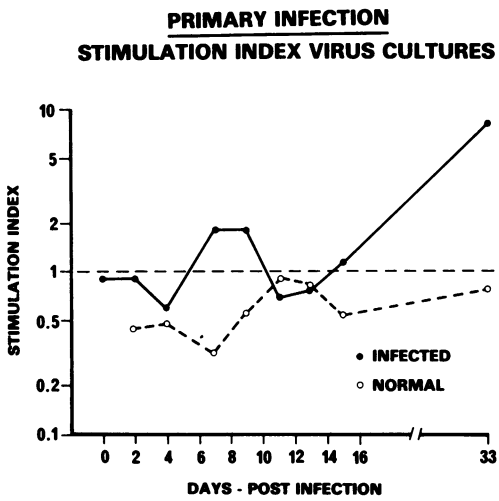

FIG. 2. *SIs for vaccinia virus-stimulated whole blood cultures from control (○) and vaccinia-infected (●) rabbits. The SI is the ratio of counts per minute in virus-stimulated cultures to counts per minute in nonstimulated cultures from the same animal.*

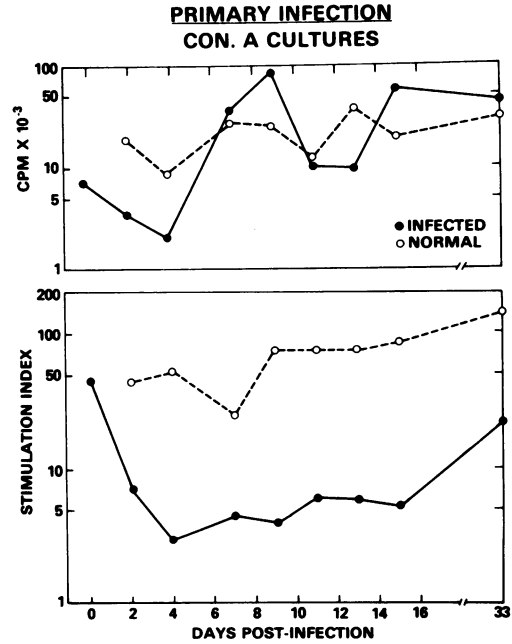

FIG. 3. *Maximal response to stimulation with concanavalin A (5 or 1 μg) of whole blood cultures from control (○) and vaccinia-infected (●) rabbits. The mean of counts per minute in the stimulated cultures is shown above with the specific SI below.*

from normal and infected animals indicates that there is no significant difference in absolute counts between the different cultures. Thus, the apparent reduced response of immune lymphocytes to concanavalin A as calculated by the SI appears to be largely an artifact resulting from the increased spontaneous thymidine incorporation in these cultures.

Leukocyte counts of blood from three infected rabbits showed an increased total leukocyte count during vaccinia infection, from 7.7 × 10^3 cells/mm³ on day 0 to 12.0 × 10^3 cells/mm³ on day 8, returning to near normal on day 14. During this period significant changes were found in the differential count characterized by a marked increase in large cells of varying

morphology, which sometimes contained small vacuoles. Preliminary studies using a stain for nonspecific esterase (20) indicate that these cells are monocytes rather than lymphocytes. The peak occurrence of these cells appeared to precede the peak in spontaneous thymidine incorporation.

Lymphocyte response to a secondary vaccinia infection. Rabbits were reinfected by intradermal inoculation with vaccinia virus 15 weeks after a primary infection. The lesions produced differed from the primary lesions in that usually no necrosis was present and local symptoms were restricted to inflammation with marked induration. The lesions persisted for varying periods and were always gone by 14 days after infection. Virus replication occurred in the lesions as demonstrated by recovery of infectious vaccinia from homogenized lesions removed 7 days after infection.

The responses of two infected and one uninfected rabbit are described. R56 showed an elevated level of spontaneous thymidine incorporation and a positive in vitro response to vaccinia virus at the time of secondary infection and had a more severe dermal reaction at the site of inoculation, with slight necrosis in some lesions. Lymphocytes from R57 showed no increase in spontaneous thymidine incorporation or response to vaccinia virus at the time of secondary infection, and less severe local reactions were observed at the sites of inoculation.

It is evident from Fig. 4 that there is a marked increase in spontaneous incorporation of thymidine by unstimulated lymphocyte cultures from rabbits exposed to a secondary vaccinia infection. An initial fall in counts was observed at day 2; this was followed by an increase in counts that reached a peak on day 9, five- to eightfold above normal cultures, and remained elevated for 35 days – the duration of the experiment. Spontaneous thymidine incorporation by these cultures at peak response was significantly less than in corresponding cultures from a primary infection.

Lymphocyte cultures from secondary infected rabbits showed a much greater response to virus stimulation than did corresponding cultures from primary infected animals. Significant increases were first measured on day 7, with levels 20- to 100-fold above cultures from noninfected animals between 8 and 18 days after infection (Fig. 4). A greater spontaneous and virus-stimulated response was found in cultures from R56 as compared with R57 at all times tested.

Presentation of these results in the form of SI showed a specific response to virus antigens as early as 5 days p.i. Significant SIs with lymphocytes from both rabbits persisted for approximately 2 weeks and returned to preboost levels by 35 days (Fig. 5).

As in the period after a primary infection, a decreased SI in response to concanavalin A was found after secondary infection (Fig. 6). This was not as marked as with lymphocytes from primary infected animals but did show a similar inverse relationship with spontaneous thymidine incorporation found in nonstimulated cultures (Fig. 6).

Macrophage response during primary and secondary vaccinia infection. Glass-adherent

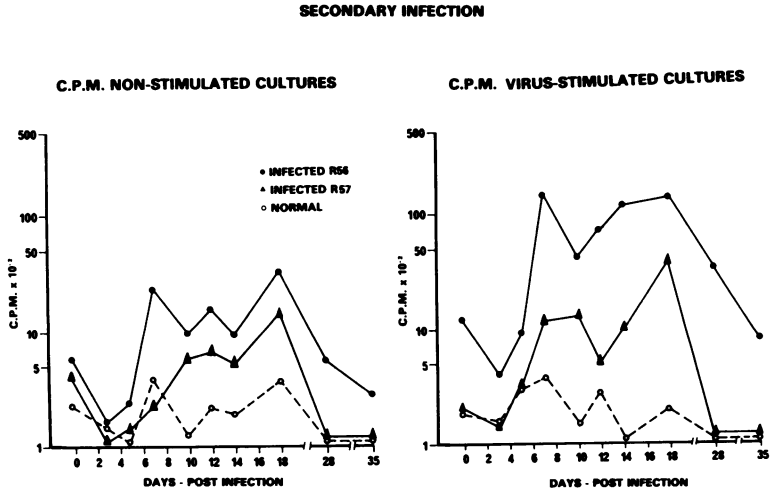

FIG. 4. *[³H]thymidine incorporation of whole blood cultures from control (○) and vaccinia-infected (R56, ●; R57, ▲) rabbits. The animals were inoculated intradermally with 10^7 PFU of vaccinia virus 15 weeks after a primary infection. The mean of counts per minute is shown for nonstimulated and for vaccinia virus-stimulated cultures.*

peritoneal macrophages from rabbits killed at different times after infection were challenged with live vaccinia virus. Macrophages from normal rabbits or those examined early after infection supported virus replication with a mean 200-fold or greater (range, 77 to 580) increase in titer (Table 1). Macrophages from rabbits killed 4 days after infection supported reduced replication, whereas those collected at 6 days or later were activated and did not support virus replication. This resistance persisted for 2 to 3 weeks; reduced levels of replication were still observed at day 56.

Macrophages collected from rabbits 15 weeks after a primary infection demonstrated an almost normal level of vaccinia replication after in vitro infection; however, activated macrophages appeared within 3 days after secondary infection of these animals (Table 1). This resistance persisted for 9 weeks, at which time the experiment was terminated.

DISCUSSION

The aim of this study was to follow the lymphocyte responses of rabbits after primary and secondary infection with vaccinia virus and to determine whether these responses were related to the appearance and resistance of activated macrophages.

The results of this study indicate that lymphocytes recovered from rabbits during the acute phase of a primary infection with the WR

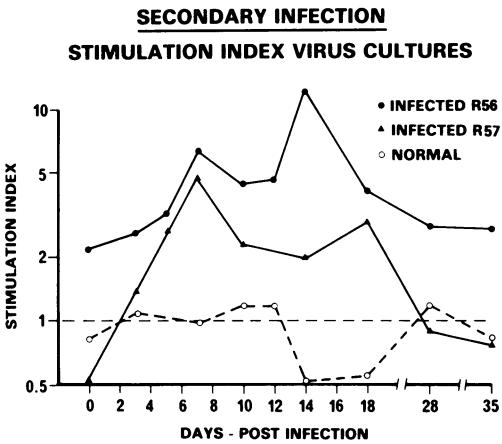

FIG. 5. *SIs for vaccinia virus-stimulated whole blood cultures from control (○) and vaccinia-infected (●, R56; ▲, R57) rabbits after a secondary infection. The SI is the ratio of counts per minute in virus-stimulated cultures to counts per minute in non-stimulated cultures from the same animal.*

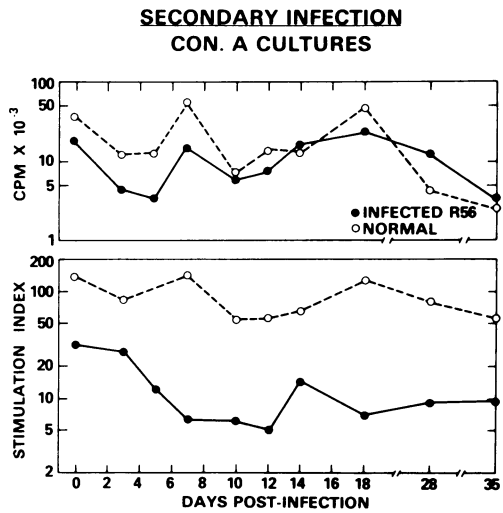

FIG. 6. *Maximal response to stimulation with concanavalin A (5 or 1 μg) of whole blood cultures from control (○) and vaccinia-infected (●, R56) rabbits after a secondary infection. The mean counts per minute is shown above, with the specific SI below.*

TABLE 1. *Replication of vaccinia virus in peritoneal macrophages*[a]

Infection	−Fold increase in virus titer[b] on day p.i.													
	0	2	3	4	6	7	8	10	11	14	17	21	56	63
Primary[c]	210[d]	290		16	0.3	2.8	1.1	8.5	6.5		33	3.0	28	
	(5)	(1)		(2)	(1)	(2)	(1)	(1)	(1)		(1)	(1)	(1)	
Secondary[e]	57		0.8			0.4			0.8	0.2			1.3	1.2
	(1)		(2)			(2)			(2)	(1)			(1)	(1)

[a] Starch-induced peritoneal macrophages were collected from individual animals at different times after intradermal infection with vaccinia virus. The glass-adherent cells were challenged in vitro with vaccinia virus and the degree of virus replication was measured.

[b] Ratio of titer at 48 h to titer at 0 h.

[c] Rabbits were infected by intradermal inoculation of 10^7 PFU of vaccinia virus.

[d] Mean increase in titer (number of rabbits).

[e] Rabbits were reinfected by intradermal inoculation of 10^7 PFU of vaccinia virus 15 weeks after a primary infection.

strain of vaccinia (i) incorporate high levels of thymidine in the absence of virus stimulation, (ii) are relatively insensitive to specific virus stimulation in vitro in that only a transient and low level SI is observed, and (iii) when SIs are compared, appear to be markedly hyporesponsive to the nonspecific mitogen concanavalin A as compared with lymphocytes from normal rabbits. In comparison to primary infections, the lymphocytes from rabbits experiencing a secondary vaccinia infection showed (i) a similar but decreased spontaneous thymidine incorporation, (ii) a greater and more prolonged specific response to virus stimulation, and (iii) a similar but less marked apparent hyporesponsiveness to concanavalin A stimulation.

Rosenberg et al. (13) reported a peak response of peripheral leukocytes to vaccinia virus 14 days after primary infection. These workers did not present any evidence of increased spontaneous thymidine incorporation by lymphocytes from infected animals, although this was a striking observation of the present study. This level of spontaneous thymidine incorporation was so great as to possibly mask any specific stimulation in response to vaccinia virus during the acute stage of the infection.

The virus-specific lymphocyte response after a secondary vaccinia infection was greater than during a primary infection, but did not appear more rapidly. In contrast, Rosenberg and Notkins (14) found a lower specific lymphocyte response to secondary herpesvirus infection compared with a primary infection. They also found that after a transient increase in stimulation levels, the in vitro lymphocyte responses fell to pre-reinfection levels, with no evidence of a lasting increased response. In this study the elevated lymphocyte response to virus stimulation lasted for approximately 2 weeks after secondary infection.

The production of activated macrophages appeared to differ in a secondary compared with a primary infection. Although some animal-to-animal variation occurred, activated peritoneal macrophages were usually only found 6 days or later after a primary infection and persisted usually for 1 to 2 weeks. In contrast, activated macrophages were present in both rabbits killed 3 days after a secondary infection and were present in rabbits killed 56 and 63 days after reinfection. The degree of resistance was also greater after secondary infection; a complete absence of challenge virus replication was usually observed, compared with the low level of multiplication found after a primary infection.

Studies in mice have produced results similar to those obtained in rabbits. After *Listeria monocytogenes* infection, North and Deissler (10) found a short-lived state of active immunity, i.e., activated macrophages and a long-lived state of increased immunological potential that enabled the host to respond to secondary infection in an accelerated manner. This immunological potential was associated with the presence of a small number of nonreplicating T cells. These workers observed a more rapid appearance of activated macrophages after a secondary infection but associated with this was a more rapid higher increase in spontaneous thymidine uptake by spleen lymphocytes in vivo.

The finding of elevated levels of spontaneous thymidine incorporation by peripheral lymphocytes after vaccinia infection of rabbits is similar to that seen after some virus infections in man. Thus, Finkel and Dent (2) reported impaired lymphocyte responses to phytohemagglutinin during acute measles infection and suggested that this might be related to elevated spontaneous deoxyribonucleic acid synthesis in leukocytes from these patients. Munyer et al. (8) followed lymphocyte responses after immunization with live measles-mumps-rubella vaccine and distinguished between a true decreased responsiveness to *Candida* antigen and an apparent decreased response to concanavalin A. As observed in the present study, the apparent decreased response to concanavalin A was an artifact due to the presentation of lymphocyte responses as SIs, calculated on the basis of greatly elevated counts obtained from "nonstimulated" cultures.

Although it is evident from this study that the in vitro lymphocyte stimulation test is a relatively insensitive measure of immune response to vaccinia infection, spontaneous thymidine incorporation by lymphocytes from infected animals was an extremely sensitive indicator of an immune response. Lymphocyte response to vaccinia infection could be detected as early as 2 days after infection by this method. Pagé et al. (12) reported that increases in spontaneous thymidine incorporation of leukocyte cultures were a good indicator of impending graft rejection in humans.

The problem of correlating macrophage and lymphocyte responses is difficult. In a primary infection there is a good temporal relationship between the appearance of activated macrophages and increased spontaneous lymphocyte stimulation, since both are first detectable at 4 to 6 days after infection and return to near normal levels after 2 to 3 weeks. This relation-

ship does not apply during a secondary infection in which a rapid appearance of activated macrophages was observed, although increased spontaneous lymphocyte stimulation was not found until 7 to 9 days after reinfection. The level of spontaneous activity was lower than in a primary infection, yet the macrophages were more resistant; the resistance and elevated spontaneous activity did, however, persist much longer after a secondary infection.

Cytophilic antibody adsorbed to the surface of macrophages is thought not to play a significant role in the observed resistance of activated macrophages to in vivo infection with vaccinia virus (15). Also, studies using herpes simplex virus as the in vitro challenge virus showed a parallel resistance to infection, as demonstrated by a reduced number of cells containing herpes simplex virus antigens demonstrable by immunofluorescence (McLaren, unpublished observations). In this instance antibodies to herpes simplex virus were absent from the vaccinia-infected rabbits, suggesting a mechanism of macrophage activation other than cytophilic antibody.

Lymphocytes exert their influence on macrophages via soluble products, such as migration inhibition factor, macrophage activating factor, and interferon. Attempts in the present study to measure serum interferon levels were inconclusive, since only marginally detectable titers were found. In vitro studies using higher levels of interferon than those found in the rabbit sera tested here, however, showed the macrophage/vaccinia system to be unaffected by pretreatment with interferon (McLaren, unpublished observations). It is therefore possible that production of lymphokines other than interferon may have produced the activation of macrophages. The preliminary finding of monocytosis at the time, or even preceding, the increased spontaneous thymidine incorporation suggests the possibility that activated circulating monocytes may produce lymphocyte-stimulation factors, assuming that the increased thymidine incorporation is due to lymphocyte blastogenesis. Other work in progress indicates that the cells responsible, directly or indirectly, for the increased spontaneous stimulation are lost by Hypaque-Ficoll fractionation. These results may thus extend previous observations on macrophage requirements for lymphocyte responses (5, 11, 19).

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