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Characterization of the effector cell from Mycobacterium tuberculosis-sensitized mice that inhibits encephalomyocarditis virus replication

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CHARACTERIZATION OF THE EFFECTOR CELL FROM *MYCOBACTERIUM TUBERCULOSIS*-SENSITIZED MICE THAT INHIBITS ENCEPHALOMYOCARDITIS VIRUS REPLICATION

By

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Peritoneal cells (PC) from mice sensitized with nonviable Mycobacterium tuberculosis inhibited encephalomyocarditis virus (EMCV) replication in mouse embryo fibroblast (MEF) monolayers. At a PC/MEF ratio of 20, viral replication was inhibited 1-2 $\log_{10}$ (90-99%) with sensitized cells as compared to PC from unsensitized mice. To determine the effector cell, PC were separated into distinct cell populations. The results showed that the effector cell adhered to plastic, nylon wool, siliconized glass, and baby hamster kidney microexudate. Populations enriched for neutrophils by Hypaque fractionation were ineffective. Likewise, T lymphocytes which did not adhere to nylon wool columns did not inhibit EMCV replication. The unimportance of functionally mature T lymphocytes in the inhibition of EMCV replication was confirmed in studies with mycobacteria-sensitized athymic (nude) mice. EMCV replication was not affected by removal of macrophages from PC populations with protein-coated carbonyl iron or destruction of macrophages with protein-coated silica. Furthermore, irradiated sensitized PC were ineffective in the inhibition of EMCV replication. Additional studies showed that the nonspecific immunity was expressed by immune type II interferon. Immune interferon production in PC cultures indicated that cell populations depleted of macrophages did not inhibit EMCV replication as well as supernatant fluids prepared from cultures of unseparated PC. In contrast, macrophage-rich cell populations produced fluids which were highly active. Thus, it appears that the B lymphocyte was the effector cell which inhibited EMCV replication by cellular cooperation with the macrophage and subsequent production of immune interferon.

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ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
BCG	Bacille Calmette Guérin
BHK	baby hamster kidney
CFW	Carworth Farms Webster
CMC	cell-mediated cytotoxicity
CMI	cell-mediated immunity
CNS	central nervous system
EDTA	ethylene diaminetetraacetic acid
EMCV	encephalomyocarditis virus
FCS	fetal calf serum
HSV	herpes simplex virus
i.c.	intracranial
i.d.	intradermal
i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
MEF	mouse embryo fibroblast
MEM	minimal essential media
ML	mouse L
NK	natural killer
NPAC	nonphagocytic adherent cell
PBS	phosphate buffered saline
PC	peritoneal cell
PFU	plaque-forming unit

RES	reticuloendothelial system
s.c.	subcutaneous
SRBC	sheep red blood cell
STV	saline-trypsin-versene

INTRODUCTION

Nonspecific resistance to infection is an important facet of immunology that has been under considerable investigation in recent years. The concept of nonspecific resistance is that a host can be exposed to an antigen, either living or dead, which elevates its resistance to subsequent unrelated challenge by pathogens or tumors. The majority of the model systems used to study nonspecific resistance involve an eliciting organism that is a facultative intracellular parasite of macrophages. Commonly used inducers of nonspecific resistance are species of Mycobacteria (29), Corynebacteria (27), Listeria (46), Brucella (16), Salmonella (39), Proprionobacteria (16), or fractionated products of these organisms. These agents have been shown to be effective in modulating the immune response to bacterial, parasitic, neoplastic or viral diseases.

One of the first reports of nonspecific resistance was that of Pullinger in 1936 (45). He demonstrated that guinea pigs infected with Mycobacterium tuberculosis had elevated resistance to challenge with Brucella abortus. Brucella abortus infections were later shown by Naka to provide similar protection against Mycobacterium tuberculosis (36). Immunity of this type could not be transferred passively with immune serum, but could be transferred with lymphocytes, hence the name cellular immunity (39). Another model of nonspecific resistance to bacterial infection involved the intraperitoneal (i.p.) injection of viable Bacille-Calmette Guérin (BCG). The BCG was effective at protecting mice up to 10 wk against intravenous (i.v.) challenge with Staphylococcus aureus (12). The most striking protective effect, however, was seen after the

i.p. or subcutaneous (s.c.) administration of nonviable BCG.

BCG also has been shown to modulate resistance to parasitic infection. Mice injected i.v. with BCG were protected significantly against sporozoite-induced rodent malaria (56). The resistance to Plasmodium berghei infection was greatest 10 days post-BCG sensitization. This effect gradually waned until day 35 when it was undetectable. In another parasitic study, mice injected i.v. with BCG were protected against i.p. or i.v. challenges of Babesia (11). Nonspecific resistance induced by BCG was similar to levels achieved by natural recovery. Furthermore, the level of resistance was found to elevate with increased intervals up to 180 days between BCG and subsequent parasitic challenge.

It also has been determined that bacterial adjuvants can nonspecifically protect animals against neoplasia. In one such study, mice injected i.v. with BCG lived significantly longer and frequently showed advanced to complete regression of s.c. implanted Ca tumors (42). The average survival time of untreated mice was 14 days, whereas mice injected 11-13 days previously with BCG survived 27 days. In the same model (42), 85-90% of untreated mice died within 2-5 days after s.c. implantation of sarcoma 180. Mice that had been injected i.v. with BCG 7 days previously showed some resistance to the tumor, and were completely resistant to tumor growth 14-67 days after sensitization.

In another example of nonspecific modulation of the immune response, Starr et al. (59) showed that the survival time of newborn mice sensitized i.p. or intradermally (i.d.) with BCG significantly increased after an i.p. injection of HSV-2. The protective effect could not be demonstrated if BCG was given 4 days or less before challenge with HSV-2.

Nonspecific resistance has been shown to be effective against many viruses in several model systems. Mechanisms for this phenomenon have been elucidated and current findings indicate that not one, but several may be involved in the enhanced resistance (40). For example, a localized effect can amplify the effector cells. In a study by Spencer et al. (58), BCG-sensitized mice which subsequently were challenged with influenza strain A virus showed such a localization. Mice sensitized i.p. with BCG were somewhat protected against intranasal (i.n.) challenge with influenza virus, but protection was enhanced if the BCG also was instilled i.n. The enhanced effect due to localization of the BCG emphasizes the role of local nonspecific immunity and the importance of an early immune response. An effect as this has been termed compartmentalization (58). The importance of compartmentalization also has been shown in the delayed hypersensitivity response to sheep red blood cells (SRBC) after s.c. BCG administration. A maximal response to s.c. injected SRBC occurred 14 days after BCG administration when both BCG and the SRBC reached the same regional lymph node. The enhanced performance of this regional lymph node was thought to have been due to its increased size and a greater opportunity to trap cells from the circulating pool of lymphocytes (33).

The recruitment of a large number of cells and an increased clearance rate by the reticuloendothelial system (RES) is another phenomenon that has been associated with nonspecific enhancement of resistance. BCG-immunization has been shown to enhance the RES for rapid clearance of carbon particles (7). The greatest clearance rate was achieved with an i.v. BCG-sensitization 2 wk post-infection (24). Such a phenomenon is

interesting from an academic viewpoint, but the enhanced clearance is not exclusively responsible for the nonspecific resistance to viral infections. For example, the compounds lipid A, glycerol trioleate, diethyl stilbestrol and estradiol accelerated the RES clearance rate, but did not affect the course of viral infections (41). Furthermore, mice immunized with BCG were protected against Babesia, and despite heightened RES activity, dead parasites were not detected in monocytes or macrophages; instead dead and dying forms were found in circulating erythrocytes (11).

To more thoroughly understand the principles of nonspecific immunity, recent investigations have focused on effector cells and how immunomodulators express their effect. It is believed that adjuvants cast a manifold effect on the immune system that affects macrophages, cell-mediated immunity and antibody production (18). Ogra et al. (40) have described the mechanism of host defense against viral infections as a combination of many nonspecific and specific components. Furthermore, in the model system of Spencer et al. (58) mentioned earlier, mice sensitized either i.p. or i.n. with BCG were protected against an i.n. challenge of influenza virus. These authors suggested that the enhanced resistance following i.n. instillation of the BCG could have been due to enhanced humoral immunity operating in conjunction with, or completely indepedent of cell-mediated immunity. If so, it appears the BCG may have accelerated the antibody response. Similarly, rabbits immunized i.v. with BCG in the model system of Larson et al. (29) were more resistant to HSV-2 infection by corneal scarification than the intravaginal or intracorneal routes. It was suggested that neutralizing antibodies produced in the

nervous system following scarification infection, in conjunction with the BCG immunization, may have had a central role in resistance. In corroboration of this suggestion, Larson and his associates (5) showed that mice administered BCG i.v. were not protected against HSV-2 challenge by the intravaginal route. If, however, the BCG-sensitized animals were inoculated i.v. with anti-HSV-2 antiserum prior to infection, a degree of protection was noted. Furthermore, it is known that humans have recurrent herpetic lesions in the presence of high levels of circulating anti-HSV antibody. If, however, similar patients are administered BCG, the recurrent infections are markedly suppressed (4). Antibodies are secreted by the B lymphocyte, but antibody production involves more than the presence of the B cell; B cells require the cooperation of T cells (10) and macrophages (35) to express specific immunity. Thus, introduction of an immunomodulator, like BCG, which has a broad effect on the matrix of immunological effector cells appears to enhance this antibody production.

Morahan et al. (34) tested C. parvum and C. acnes as modulators of nonspecific resistance in mice against HSV-2. Mice were treated i.p. with either heat-killed C. parvum or C. acnes and then challenged i.p. on day 14 with HSV-2. Both C. parvum and C. acnes significantly protected mice against death; neither, however, had an effect on survival time after an intravaginal HSV-2 challenge. Treatment of the C. parvum-stimulated mice with silica, a purported macrophage toxin, greatly increased their susceptability to i.p. injected HSV-2. In contrast, similar treatment of C. acnes-injected mice did not inhibit the antiviral activity, which suggested that different mechanisms of nonspecific

resistance were responsible for the enhanced protection. Reduction of resistance after silica treatment was associated with early and sustained viral replication in the visceral organs due to the killing of activated macrophages. These activated macrophages can be identified by their enhanced potential for phagocytosis and their accelerated ability to spread on a glass surface (39). Morphologically, these phagocytes have more lysosomes (39) containing increased amounts of lysozymes (8). Recently, activated phagocytes have been more precisely described by specific biochemical changes in cellular components, enzymes, metabolism and secretions (26).

In another example of nonspecific modulation of the immune response to viral disease, the i.p. injection of Corynebacteria parvum was shown to enhance the resistance of newborn mice injected intracranially (i.c.) with Junin virus (9). No protection was detected if the Corynebacteria injection preceded the virus, some protection was noted if Corynebacteria followed the virus, and the greatest degree of resistance developed when C. parvum and virus were administered simultaneously. Furthermore, neither antiviral antibody or interferon were associated with protection, and silica dust did not abrogate the resistance. C. parvum was believed to suppress T lymphocyte functions because unsensitized thymectomized mice were not susceptable to Junin virus, whereas, the transfer of spleen cells to these thymectomized mice restored their vulnerability. C. parvum suppression of T cell function correlated with the finding that thymuses of C. parvum-sensitized mice were reduced in size. T lymphocytes also have been shown to participate in cell-mediated cytotoxicity (CMC). In a model system of specific immunity, mice which were

not immunomodulated (25) were injected i.p. with mouse adenovirus. Seven-10 days later their splenocytes were used as effectors against mouse adenovirus-infected mouse embryo fibroblasts (MEF) or mouse-L (ML) cells. Studies indicated that treatment with antimouse immunoglobulin plus complement or plastic adherence eliminated the B cell and macrophage, respectively, and did not eliminate cytotoxicity. Cytotoxicity was significantly reduced, however, after splenocytes were treated with antimouse thymocyte or anti-θ serum plus complement.

Natural killer (NK) cells have been associated with nonspecific resistance to viral infections (60). This cell is nonadherent, nonphagocytic and unique because it lacks demonstrable Fc-receptors and θ-antigen on its plasma membrane (64). Furthermore, the NK cell has been demonstrated in nude mice which lack conventional mature T lymphocytes (63). One of the first reports of NK cell activity against virus-infected target cells was by Anderson (3). Spleen cells harvested from mice which had not received an immunomodulator had an innate ability to lyse Sendai virus-infected L-cells. Antibody was not involved in destruction of the target cell since lysis occurred with cells that had been depleted of B lymphocytes by nylon wool adherence. Furthermore, the removal of macrophages and other cells bearing complement receptors by Ficoll fractionation of cells complexed with erythrocytes had no effect on the ability of the remaining cells to lyse virus-infected targets. Elimination of T lymphocytes by thymectomy, anti-θ plus complement, or x-irradiation with subsequent bone marrow reconstitution did not decrease the cytotoxicity. These studies suggested that the effector cell resembled a NK cell.

In the model system developed by Tracey et al.(60), i.p. immunization of mice with BCG gave rise to peritoneal cells that were lytic against a broad range of target cells, as well as normal and malignant tissue cell lines. The lytic NK cells which were demonstrable within hours of BCG administration produced a maximal effect on day 4 or 5, and then decreased in activity to control levels after 20 days. In a similar study by Herberman et al. (22), NK cell activity in mice immunized i.p. with C. parvum was enhanced against tumor cells.

The previous examples of target cell lysis by NK cells did not require antibody, but destruction of virus-infected cells by K cells has been attributed to antibody-dependent cellular cytotoxicity (ADCC). In the nonelicited ADCC system of Shore et al. (62), it was determined that human blood lymphocytes incubated in the presence of HSV-1 antisera enhanced the cytotoxicity of HSV-1 infected Chang liver cells as compared to similar lymphocytes incubated with normal serum. Pretreatment of the lymphocytes with carbonyl iron, nylon wool or adherence to plastic did not ablate the ADCC, nor did removal of the rosette-forming cells (T cells). These data, and the presence of the characteristic surface Fc-receptors indicated the effector cell was the K cell. Mac Farlan et al. (32) demonstrated that normal peritoneal mouse cells were cytotoxic to Semliki Forest virus-infected P815 mastocytoma cells in the presence of antiviral antibody. In contrast to the studies by Shore et al. (62), the effector cell was adherent to nylon wool, removed by carbonyl iron and sensitive to both silica and carrageenan. Furthermore, treatment with trypsin, ethylene diaminetetraacetic acid (EDTA), and anti-θ plus complement did not inactivate the effector. The authors suggested that

these results unequivocally indicated that the effector cell expressing ADCC was the macrophage.

A different effector cell has been isolated in a study by Nathan et al. (38). In this study, mice were sensitized i.p. with BCG and PC were harvested up to 20 days post-BCG injection. The PC were allowed to adhere to plastic for 2-3 h; the adherent cells which did not pellet in a Ficoll gradient were harvested and determined to be 4% phagocytic. Incubation of these cells with tumor cells for 2 days showed a marked inhibition of MCA-3 tumor activity. The cell isolated by Nathan et al. was present in peritoneal washings in concentrations of 6% and 18%, respectively, in normal and BCG-immunized animals (37). The cell has been termed a nonphagocytic adherent cell (NPAC). In addition to these properties it is esterase positive and has neither Fc-receptors or θ-antigens. The NPAC appears to be an intermediate cell, but has tentitively been termed a B cell subtype. The discovery of the NPAC is important because, in the past, the property of adherence has been used as a criteria for identification and isolation of macrophages. The authors speculated that in previous studies, macrophages may have been unknowingly contaminated with the NPAC, and the effects attributed to the macrophage may actually have been a function of the NPAC.

Cells active in antiviral immunity also have been credited with the production of lymphokines which act as mediators of resistance. In a representative study, Sonnenfeld et al. (57) selectively eliminated in vivo various cell populations to determine the cell involved in the production of type II interferon. Type II interferon was produced in mice that had been sensitized i.v. with BCG cell walls and challenged i.v. 4

wk later with old tuberculin. The s.c. injection of antithymocyte serum or the i.v. administration of cyclophosphamide, a B cell immunosuppressive drug, into the sensitized mice decreased the titer of type II interferon. X-irradiation also eliminated type II interferon production, but production could be initiated again by reconstituting the x-irradiated mice with B cells and then T cells. Mice reconstituted with B cells alone did not produce interferon, and the T cells had to be present for 11 days before interferon could be detected.

In an interferon study by Gresser et al. (20), it was determined that mice injected s.c. or i.p. with encephalomyocarditis virus (EMCV) died of central nervous system (CNS) disease on the fourth or fifth day post-infection. EMCV is a member of the RNA picornaviridae family (17) and is a known endogenous murine pathogen (18). Interferon was present in animals infected with EMCV. To determine the importance of interferon, mice were administered sheep antimouse (type I) interferon just before i.p. or i.c. infection with virus. With this treatment the mice became ill by 24 h, were moribund by 36 h without CNS involvement and nearly all mice died by 48 h. In control-infected mice, it was difficult to detect EMCV in serum or visceral organs, but high titers of EMCV developed in the brain. In contrast, anti-interferon-treated mice had high visceral organ EMCV titers and appeared to die of an overwhelming systemic infection. The authors suggested that interferon was as early defense mechanism in that it prevented the spread of EMCV in visceral organs. Anti-interferon destroyed this barrier, and mice died of a systemic infection before virus spread to the CNS.

As the studies mentioned above have indicated, immunity to viral

agents is not limited to the involvement of a specific cell or single mechanism. The expression of viral immunity is as varied as the challenging virus. The model system of nonspecific immunity that I have addressed is that of Lodmell and Ewalt, who determined that female C57BL/10ScN mice sensitized i.p. or i.v. with nonviable Mycobacterium tuberculosis suspended in an oil-in-saline emulsion were markedly resistant against i.v., i.p., s.c. or i.m. EMCV challenge (30). Interferon was not detected in sera of the mycobacteria-sensitized mice. Furthermore, less than 50% of the mice that survived EMCV challenge had demonstrable EMCV neutralizing antibody. This lack of specific antibody suggested that protection was due to neither antibody nor ADCC. Protection dependent upon T lymphocytes also was excluded because neonately thymectomized or athymic nude mice sensitized with nonviable mycobacteria were resistant to EMCV (31). Interestingly, nonspecific protection was abrogated after treatment with the immunosuppressive drug cyclophosphamide, or the purported macrophage toxin, silica dust (31).

More recently, an in vitro model has been developed (Pusateri et al., accepted for publication) to study in greater detail the nonspecific immunity to EMCV elicited by peritoneal cells from mycobacteria-sensitized mice. The investigation which I propose, is to isolate and characterize the cell(s) that is responsible for the in vitro inhibition of EMCV replication. To accomplish this, techniques which separate various cell populations by their physical and physiological properties will be used. These separated cells will be tested in viral inhibition assays to determine their effect on EMCV replication. From the previous in vivo studies with this model system (30,31), it appeared that the macrophage

may have had a role in the nonspecific immunity to EMCV. For this reason, the investigation will begin by examining adherent properties of the effector cell. Surfaces to enrich for adherent macrophages will include baby hamster kidney microexudate, siliconized glass flasks, and teflon. Nylon wool, silica dust, plastic flasks and carbonyl iron will be used to deplete macrophages. Nylon wool columns, in addition to studies with athymic nude mice, will determine the importance of the T lymphocyte. Fractionation of neutrophils will be accomplished with Hypaque-density gradients. These techniques, and others as needed, will determine the characteristics of cell(s) from mycobacteria-sensitized mice that are responsible for the in vitro inhibition of EMCV replication.

Experiments reported herein indicate that the PC responsible for inhibition of EMCV replication was adherent to plastic, BHK microexudate, siliconized glass and nylon wool. PC populations enriched for neutrophils by Hypaque fractionation were ineffective. Studies with nylon wool and athymic nude mice demonstrated the unimportance of the functionally mature T lymphocyte. PC depleted of macrophages by either phagocytosis of protein-coated carbonyl iron and treatment with magnetic force, or ingestion of silica inhibited EMCV replication. Furthermore, irradiated PC were ineffective. Other studies indicated that an antiviral mediator was responsible for the inhibition of EMCV replication. PC cultures depleted of macrophages did not produce as effective a mediator as PC populations containing macrophages. The data suggests that the effector cell was the B lymphocyte which cooperated with the macrophage to produce the antiviral mediator.

Mice. Female 1-3 month old C57BL/10ScN mice, or 1-3 month old male and female BALB/c (nu/+) or athymic (nu/nu) mice produced by successive cross-intercrossings onto a BALB/c background were used unless otherwise noted. The BALB/c (nu/+) and athymic (nu/nu) mice were maintained in a separate animal room in sterile cages with filter caps. All bedding, food and water was autoclaved. Embryos of Carworth Farms Webster (CFW/R) mice were used to make mouse embryo fibroblast cultures, and the stock pool of encephalomyocarditis virus was prepared in 19-21 day old Swiss Webster mice. All mice were reared from stocks maintained at the Rocky Mountain Laboratories, Hamilton, MT.

Mycobacteria. Acetone-dried Mycobacterium tuberculosis, strain Jamaica was obtained from Dr. Carl Larson, University of Montana, Missoula, MT. The culture was isolated from a fatal case of tuberculosis in 1933 by J. Freund and E. Opie (personal communication, George Kubica, Center for Disease Control, Atlanta, GA).

Mycobacteria oil-in-saline emulsion. Twelve and one-half mg of acetone-dried mycobacteria was autoclaved and then heat-dried overnight. The mycobacteria were placed in a sterile tissue grinder (Scientific Glass Apparatus Co., Inc., Bloomfield, NJ) and ground with 4 drops (approximately 160 µl) of light mineral oil (Drakeol 6VR, Pennsylvania Co., Butler, PA) to a smooth paste with a teflon pestle powered by a drill press rotating at 800 RPM. After grinding, the tissue grinder containing the paste was heated in a 75°C water bath for 10 min. 5 ml

of heated (75°C) 0.15 M NaCl containing 0.2% Tween 80 was then added to the tube and the grinding continued another 5 min. This emulsion was heated to 65°C for 30 min, ground for another 5 min and then examined microscopically to insure incorporation of the mycobacteria with the oil. 5-6 wk old female mice were injected i.p. with 0.2 ml of the mycobacteria emulsion (500 μg/mouse) for nonspecific sensitization. Control mice were injected with the emulsion without mycobacteria or were used untreated.

Media. Dulbecco's modified phosphate buffered saline (PBS) used for washing cells and monolayers was prepared as follows: 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, and 0.1 g $MgCl_2 \cdot 6H_2O$ were dissolved in 1000 ml distilled H_2O and filter sterilized. For the nylon wool procedure, PBS without Ca^{++} and Mg^{++} was prepared.

The standard media for tissue culture growth was Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, NY) supplemented with 10% heat inactivated fetal calf serum (FCS) (GIBCO, Grand Island, NY), 200 IU of penicillin G (Eli Lilly and Co., Indianapolis, IN) per ml, and 1 μg of amphotericin B (E.R. Squibb and Sons, Inc., Princeton, NJ) per ml (MEM-10). The FCS concentration was adjusted to 20% (MEM-20) for freezing cells in liquid nitrogen or to 2% (MEM-2) for viral inhibition assays.

The overlay media methocel, for virus titration assays, was prepared as follows: 3 g methylcellulose (4000 centipoise, Fisher Scientific, Fair Lawn, NJ) and 200 ml of distilled water were separately autoclaved and then combined. This mixture was heated to 60°C, stirred

for 2 h and then the heat was discontinued and the stirring mixture was allowed to cool to 40-45°C. 200 ml of 2X MEM-10 was added to the cooled methylcellulose and stirring was continued an additional 2 h. The methocel was stored at 4°C overnight and vigorously agitated prior to use.

Peritoneal cells. Peritoneal cells were harvested from mice following cervical dislocation and the i.p. injection of 5 ml of PBS containing 5 units of heparin/ml. After soaking the mice with 70% ethanol, a midsection of skin was cut free to expose the peritoneal wall which was punctured. The heparinized PBS containing the PC was collected in a 50 ml polypropylene centrifuge tube (Corning Glass Works, Corning, NY). The fluid volume in the tube was brought up to 40 ml with heparinized PBS and the PC were centrifuged for 6 min at 300 g. The cells were rewashed with PBS and any red blood cells were lysed by treatment at room temperature for 5 min with 5 ml of prewarmed (37°C) pH 7.2 Tris buffer containing 0.83% NH_4Cl. The PC were washed again with PBS and resuspended in appropriate media at the desired cell concentration.

Tissue culture. Mouse embryo fibroblasts were prepared from CFW/R mouse embryos. Immediately after removal, the embryos were placed in heparinized PBS at 4°C and under sterile conditions they were clipped free of heads, legs and tails. The remaining torsos were sectioned with scapels and then stirred for 18-24 h at 4°C in a 200 ml trypsinizing flask containing 100 ml of PBS supplemented with 0.25% trypsin (DIFCO Laboratories, Detroit, MI) and 2 ml of FCS. After trypsinization, the cell suspension was passed through sterile gauze into three 50 ml tubes,

centrifuged at 300 g for 10 min, washed twice in 40 ml of MEM-10, treated with warm Tris buffer containing 0.83% NH_4Cl and resuspended in MEM-10 to approximately 10^7 cells/ml. One ml of the cell suspension was added to 150 cm^2 plastic flasks (Corning Glass Works, Corning, NY) containing 30 ml of MEM-10. The flasks were incubated at 37°C with 5% CO_2 in air (standard conditions) until monolayers were established.

The established cell lines used in this study were mouse-L obtained from Dr. R.K. Gerloff, Rocky Mountain Laboratories, Hamilton, MT and baby hamster kidney-21 (BHK) (GIBCO, Grand Island, NJ). Both lines were grown by seeding 150 cm^2 plastic flasks with 10^7 cells from the preceding passage and then incubating at standard conditions until monolayers developed.

Monolayers of primary and established cell lines were removed for freezing by washing the cells with 15 ml of PBS and then incubating them at 37°C for 10 min with 35 ml of saline-trypsin-versene (STV). The formula for STV is as follows:

NaCl	8.0 g
KCl	0.4 g
dextrose	1.0 g
$NaHCO_3$	0.58 g
trypsin	0.5 g
versene	0.2 g
phenol red	0.9 ml of 0.5% solution
glass distilled H_2O	1000 ml

filter sterilize

The trypsinized cells were frozen for future use as follows: cells were collected in 50 ml centrifuge tubes containing 5 ml of FCS, centrifuged at 300 g for 10 min, and washed twice in MEM-10. The pelleted

cells were diluted to 4 ml/flask monolayer in a solution that contained 10% dimethyl sulfoxide (J.T. Baker Chemical Co., Phillipsburg, NJ) and 90% MEM-20. One ml aliquots of the cell suspension were sealed in 1.2 ml cryules (Wheaton Scientific, Milliville, NJ) with a hydrogen-oxygen torch. The sealed cryules were allowed to cool at a controlled rate overnight in a 95% ethanol bath held at -70°C and then placed on pre-cooled aluminum canes and submerged in liquid N_2 for prolonged storage.

EMCV. EMCV was obtained from Dr. Michael Ross, National Institutes of Health, Bethesda, MD. A stock pool of EMCV was prepared by the intracerebral injection of 19-21 day old Swiss Webster mice with $2x10^3$ plaque-forming units (PFU) in 0.03 ml. The brains were harvested from infected mice 24 h later, pooled, and homogenated in a blender. 1 ml aliquots of the brain homogenate were placed in vials and stored at -70° C. Before use, the brain homogenate was centrifuged at 1800 g for 5 min. The stock pool titered $7.0x10^7$ PFU/ml on ML cells.

Virus inhibition assay. MEF monolayers in TC-96 tissue culture plates (2.8 mm^2 well surface, Linbro Scientific, Inc., Hamden, CT) were infected for 2 h by incubation with 0.03 ml of MEM containing 2-4 PFU of EMCV. After adsorption, the EMCV inoculum was aspirated and the monolayers were washed once with PBS. The PC were overlayed on the infected monolayers at a concentration of $6.0x10^5$ cells in 0.15 ml of MEM-2 (20:1 PC/MEF cell ratio) unless otherwise noted. Each cell preparation was tested in quadruplicate wells. 18 h post-infection monolayers were detached by scraping the well with the tip of a 1 ml plastic pipette

(Falcon, Oxnard, CA). Entire cultures then were harvested and frozen at -70°C until titrated for infectious virus.

Virus titrations. Serial ten-fold dilutions of the virus preparations were performed with 4°C MEM. Duplicate ML monolayers grown in TC-24 wells (20 mm^2 surface, Linbro Scientific, Inc., Hamden, CT) then were infected for 2 h with 0.2 ml of each dilution. The fluid was aspirated, and the monolayers were washed once with PBS and overlayed with 0.5 ml of prewarmed (37°C) methocel. The titration was incubated for 20-24 h, the methocel aspirated and the monolayers washed with PBS. Monolayers were fixed for 5 min with 95% ethanol and subsequently stained with a 1:5 dilution in distilled water of a giemsa stain stock solution (0.8 g giemsa, 50 ml methanol, and 50 ml glycerol) for 30 min to contrast viral plaques.

Preparation of supernatant fluids from PC cultures. PC suspended in MEM-2 at 4.0×10^6 PC/ml were incubated at standard conditions for 24 h in TC-24 wells. Supernatant fluids were harvested, centrifuged and frozen at -30°C until used. For determination of antiviral activity, 0.15 ml of the supernatant fluids was incubated on each of 4 EMCV-infected MEF monolayers grown in TC-96 wells in lieu of effector cells as in the previously mentioned conventional viral inhibition assay. The titration of EMCV was performed in the ML TC-24 system.

Preparation of siliconized glass. Various glass articles were siliconized by immersion for 1 min in a siliconizing solution (Prosil-28,

PCR Research Chemicals, Inc., Gainesville, FL) diluted 1:100 in distilled H_2O. The articles were drained and allowed to stand for 15 min in tap water and then distilled water for a similar period of time. Prior to use, the glass was dried and sterilized in the autoclave.

Cell separation by phagocytosis of protein-coated carbonyl iron. Protein-coated carbonyl iron enhances the selectivity of phagocyte separation by eliminating adherence of nonphagocytic cells that may adhere to untreated carbonyl iron. 25 mg of carbonyl iron (SF grade, GAF Corp., New York, NY) was incubated at standard conditions for 1 h in a siliconized glass test tube with 1.0 ml of MEM consisting of 50% heat-inactivated calf serum. The carbonyl iron preparation then was mixed with 10^8 pelleted PC and incubated at standard conditions for 45 min. Following incubation, the tube containing the protein-coated carbonyl iron and cells was treated for 1 min with lateral magnetic force; the iron and nonphagocytic cells that had phagocytized iron were drawn to the side of the tube so that the cells could be collected and transferred to another siliconized tube. These cells were centrifuged and the pelleted cells were treated again with a similar protein-coated carbonyl iron preparation for the same period of time. The nonphagocytic cells were separated in the same fashion as the first treatment, and then were suspended in MEM-2 for testing in the viral inhibition assay. Cell viability of the nonphagocytic cells always exceeded 90%.

Cells separated by nylon wool. New or used nylon wool (Fenwal Laboratories, Deerfield, IL) was soaked in 0.02% sodium azide in PBS,

rinsed 5 times in distilled water, and then boiled in 10 mM EDTA for 30 min. The wool was rinsed 10 times per day with 3-4 liters of distilled water for 5 days. After the final rinse, the water was wrung out by hand and the wool was allowed to dry for 3 days at 37°C. After drying, the wool was fluffed by pulling it apart wisps at a time and then packed into 10 ml syringes to a compressed volume of 5 cc.

The nylon wool column was mounted on a stand, and as described (51), 100 ml of 37°C MEM-10 was allowed to drip through the wool. The column then was plugged and incubated for 2 h at standard conditions. After equilibration, up to 1.0×10^8 PC in 1 ml of MEM-10 was allowed to settle into the wool at 37°C; one ml of MEM-10 preheated to 37°C was added to the column after 15 min and 30 min of incubation. At 45 min of incubation, the nonadherent cells were eluted from the column over a 25 min period with 20 ml of MEM-10 preheated to 37°C. The adherent cells were teased from the wool with forceps in 30 ml of PBS without Ca^{++} and Mg^{++}; during the teasing, 20 ml of MEM-10 was slowly added to the PBS. The adherent and nonadherent cells were centifuged and resuspended in MEM-2. Nylon wool effluent cells were approximately 85% viable and the adherent cells were 70% viable.

Cell separation by adherence to BHK microexudates and siliconized flasks. (A) Preparation of substrates. BHK-21 monolayers in 25 cm^2 plastic flasks (Corning Glass Works, Corning, NY) were treated for 10 min at 37°C with 5 ml of 10 mM EDTA; the EDTA was decanted and the flasks rinsed twice with PBS. The cellular material that remained attached to the flask was the BHK microexudate. Microexudates were made

on the day of experimentation and were not held at 4°C as previously described by Ackerman and Douglas (1). Siliconized glass flasks were prepared as described earlier.

(B) Cell separation. 2.5×10^7 PC suspended in MEM-20 (3 ml for french squares or flasks) were allowed to adhere at standard conditions for 30 min. The nonadherent cells, as well as the adherent cells which became detached after 2 vigorous washes with PBS, were discarded. An EDTA solution comprised of 2 ml of 10 mM EDTA and 5 ml MEM-10 prewarmed to 37°C (4.6 ml for french squares and 2.3 ml for 25 cm^2 plastic flasks) then was incubated with the adherent cells at standard conditions. Five ml of MEM-10 subsequently was added to the flasks and the cells were gently detached by scraping with a rubber policeman. The detached cells were collected and combined with the cells in the MEM-10 that was used to rinse the flasks. The adherent cells recovered from the BHK microexudate and siliconized glass flasks were approximately 90% viable.

Isolation of cells not adherent to plastic flasks and removal of cells adherent to plastic flasks by lidocaine. 4.0×10^7 PC suspended in 5 ml of MEM-20 were placed in a 75 cm^2 plastic flask (Corning Glass Works, Corning, NY) for 1 or 2 h of adherence at standard conditions. Nonadherent cells in the MEM-20 were collected and pooled with the two subsequent 10 ml PBS flask rinses. The adherent cells were treated on ice for 30 min with 8 ml of 24 mM lidocaine (Astra Pharmaceutical Products, Inc., Worcester, MA) in PBS without Ca^{++} and Mg^{++}. Gentle scraping with a rubber policeman lifted the adherent cells which were collected and pooled with the 2 PBS rinses of the flask. Both nonadherent and

adherent cells were centrifuged and washed twice with 40 ml of PBS. Nonadherent and adherent cells were >90% and 10% viable.

X-irradiation of PC. $1.5x10^7$ PC suspended in 20 ml of MEM-10 were placed in siliconized french squares and subjected to 2000 r or 5000 r with a G.E. Maximar II. After treatment the cells were washed twice with 50 ml of PBS and suspended in MEM-2. Prior to the viral inhibition assay irradiated cells were >90% viable.

Viral neutralization. Supernatant fluids prepared from irradiated PC were combined with equal volumes of a 40 PFU/0.2 ml EMCV MEM dilution (20 PFU/0.2 ml final concentration). This viral suspension was incubated at 37°C for 1 hour, then 0.2 ml of each sample was overlayed on duplicate TC-24 ML monolayers. Suspension aliquots were adsorbed for 2 h, aspirated, rinsed with PBS and overlayed with 0.5 ml of 37°C methocel. Infected ML monolayers were incubated at standard conditions for 24 h then aspirated, rinsed with PBS and fixed for 5 min with 95% ethanol. For contrast, fixed monolayers were stained with giemsa stain for 30 min.

Destruction of phagocytes by ingestion of protein-coated silica. Dorentrop Quartz DQ 12, <5 μ diameter, was suspended at 1 mg/ml in MEM-10, sonicated for 2 min and held at 4°C overnight. Prior to use, the silica was sonicated for 1 min and then 0.1 ml of the sonicated stock suspension was added to 10^7 PC in 10 ml of MEM-10 in a 50 ml polypropylene centrifuge tube. The cells and silica were incubated for 24 h

at standard conditions. After incubation, the cells were pelleted and washed twice in 50 ml of PBS. Control cells were 64% viable and silica-treated cells were 54% viable.

Determination of phagocytic cells by latex ingestion. $5.0x10^5$ PC in 0.5 ml of MEM-2 was mixed in a siliconized test tube with 0.5 ml of latex particles suspended in heat-inactivated calf serum; the latex particles, as provided by the manufacturer (Dow Chemical Co., Indianapolis, IN), were diluted 1:25 in heat-inactivated calf serum prior to use. The cells and latex were incubated at 37°C for 1 h, resuspended in 5 ml of PBS and centrifuged at 300 g. Pelleted cells were suspended in 0.5 ml of PBS, and centrifuged at 300 g (Cytospin, Shandon Southern Products Ltd., Astmoor, Runcorn, Cheshire, England) directly on to 75 x 25 mm microslides and allowed to air-dry.

Cell differentiation by Wright's stain. Wright's stain stock was prepared by thoroughly grinding 1.0 g of Wright's stain powder with a mortar and pestle; 50 ml of methanol was slowly added to the ground powder while gentle grinding continued. When the powder had completely dissolved in the methanol, 50 ml of glycerol was added and mixed well with the methanol dissolved stain. Each batch of new stain was prepared as follows: 4 ml Wright's stain stock, 3 ml acetone, 2 ml 6.5 pH PBS, 31 ml distilled water. Slides were fixed for 2 min in 70% ethanol. After air-drying, the slides were stained in freshly prepared Wright's stain for 7 min and rinsed once with running tap water. Air-dried slides were ready for examination.

Monocyte identification with the nonspecific esterase stain. Untreated cells or cells containing ingested latex were centrifuged directly onto microslides with the Cytospin centrifuge. After the cells had air dried they were fixed for 30 sec with a pH 6.6 acetone formaldehyde solution at 4°C (20 mg Na_2HPO_4, 100 mg KH_2PO_4, 30 ml distilled H_2O, 25 ml formaldehyde (30%), and 45 ml acetone), rinsed 4 times in distilled water and then air-dried for 30 min as described by I.R. Koski et al. (28). The nonspecific esterase stain was prepared as follows:

a) α-naphthyl butyrate solution: 1 ml α-naphthyl butyrate (Sigma Chemical Co., St. Louis, MO, N-8000), and 50 ml dimethyl foramide (Sigma Chemical Co., D-4254) (Stored dark and in the freezer.)

b) Methyl green counterstain (0.5%); 500 mg methyl green (Fisher Scientific, Fair Lawn, NJ), 100 mg distilled H_2O (Stored at 4° C.)

c) Pararosaniline solution: 1 g pararosaniline hydrochloride (Gurr High Wycombe, Bucks, England), 25 ml 2N HCl (Stored at 4°C.)

d) 4% sodium nitrite solution; 100 mg sodium nitrite, 2.5 ml distilled H_2O (Prepared fresh each time.)

e) M/15 Sorenson's phosphate buffer (pH 6.3): 2.128 g Na_2HPO_4, 6.984 g KH_2PO_4, 1000 ml distilled H_2O (Stored at 4°C.)

Using fluted filter paper, 1 ml of the pararosaniline solution was filtered. This filtered solution was mixed with an equal volmme of freshly prepared 4% sodium nitrite for hexazotization. The mixture stood for at least 1 min prior to using. Then, in sequence, 44.5 ml M/15 phosphate buffer, 0.25 ml hexazotized pararosaniline, 3.0 ml α-naphthyl butrate solution were mixed and filtered through fluted filter paper into a Coplin jar. This solution was only used for one

treatment and then discarded. Slides were stained by incubating them at 37°C with the staining solution for 45 min. Slides were rinsed 4 times in distilled water and then counterstained for 30 sec with 0.5% methyl green. After rinsing and air-drying, the slides were covered with a glass coverslip and Permount. Cells examined at 400X which were intensely stained red were scored as esterase positive. All other cells were considered esterase negative.

EXPERIMENTAL RESULTS

Inhibition of EMCV replication by PC from mycobacteria-sensitized mice. The in vitro viral inhibition assay as performed by Lodmell and Ewalt was done in MEF monolayers grown in plastic tissue culture plates consisting of 20 mm^2 wells (unpublished observations). This well size was sufficient until effector cell separation techniques were utilized. Because of low cell recoveries and the necessity of a PC/MEF ratio of 20, it was necessary to miniturize the assay. The data in Table 1 show that monolayers grown in wells 2.8 mm^2 (seven times smaller than the original well) were adequate to demonstrate viral inhibition; PC at a PC/MEF ratio of 20, from mycobacteria-sensitized mice inhibited EMCV replication by 2.3 $\log_{10}$ (>99%), as compared to PC from normal mice. All subsequent assays were performed in monolayers grown in 2.8 mm^2 wells using a PC/MEF ratio of 20. The PC were harvested from mice 2-4 wk after i.p. sensitization with 500 µg of nonviable mycobacteria.

Inhibition of EMCV replication with BHK microexudate adherent PC from mycobacteria-sensitized mice. Initial in vivo work with mice sensitized with nonviable mycobacteria suggested that their resistance to EMCV involved the macrophage (31). Thus, the first attempt to identify the effector cell in PC utilized the property of macrophage adherence. It initially was determined that the mycobacteria-sensitized PC adhered to plastic so tenaciously that it was difficult to recover sufficient numbers of viable cells to use in a viral inhibition assay. If, however, adherence was done on a plastic flask covered with a BHK microexudate, 97% of the recovered cells were viable.

TABLE 1. Inhibition of EMCV replication with PC from mycobacteria-sensitized mice[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice	3.7	2.3
Unsensitized mice	6.0	none
None (media only)	6.0	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 μg of nonviable M. tuberculosis. The PC were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unsensitized and mycobacteria-sensitized PC.

The data in Table 2 show that PC which adhered to the microexudate were enriched for the effector cell in that they inhibited EMCV replication 2.6 $\log_{10}$ (>99%), as compared to 1.8 $\log_{10}$ with the unseparated PC from mycobacteria-sensitized mice; only 7% of the PC that were added to the microexudate were recovered in the adherent population. The unseparated mycobacteria-sensitized PC were 40% phagocytic by latex ingestion whereas cells adherent to the BHK microexudate were 68% phagocytic. PC that did not adhere to plastic were only 4% phagocytic and did not inhibit EMCV replication (Table 2).

Inhibition of EMCV replication with siliconized-glass adherent PC from mycobacteria-sensitized mice. Tests involving siliconized-glass surfaces also indicated that the effector cell was adherent (Table 3). PC from mycobacteria-sensitized mice which adhered to the siliconized-glass inhibited EMCV replication 2.2 $\log_{10}$, whereas the unseparated, sensitized PC inhibited viral replication by 1.8 $\log_{10}$. Only 8% of the PC that were added to the siliconized flasks were recovered in the adherent population; 86% were viable. Again, PC that did not adhere to plastic had little effect on virus replication (0.1 $\log_{10}$ inhibition). Adherent cells recovered from siliconized-glass were 65% phagocytic, whereas the untreated mycobacteria-sensitized PC were 40% phagocytic.

Different surfaces such as waxpaper, paraffin, untreated glass, cellophane, teflon, and aluminum foil also were used to separate adherent cells from nonadherent cells; none surpassed the results achieved with BHK microexudates and siliconized-glass. Furthermore, when lidocaine was used to anesthetize the adherent cells so that they could be more

TABLE 2. Inhibition of EMCV replication with baby hamster kidney microexudate adherent PC from mycobacteria-sensitized mice[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	4.1	1.8
Baby hamster kidney microexudate adherent	3.3	2.6
Plastic flask nonadherent	5.8	0.1
Unsensitized mice		
Unseparated	5.9	none
None (media only)	6.3	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 μg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were incubated for 30 min at standard conditions on flasks containing the microexudate. After incubation, the nonadherent cells were discarded and the adherent cells were removed by a 30 min incubation with 37°C EDTA, followed by gentle scraping with a rubber policman . The nonadherent cells were PC that did not adhere to plastic after a 1 h incubation at standard conditions. The peritoneal cells were incubated at a PC/MEF ratio of 20

(6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice with the other test groups.

TABLE 3. Inhibition of EMCV replication with siliconized glass adherent PC from mycobacteria-sensitized mice[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	4.1	1.8
Siliconized glass adherent	3.7	2.2
Plastic flask nonadherent	5.8	0.1
Unsensitized mice		
Unseparated	5.9	none
None (media only)	6.3	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were incubated for 30 min at standard conditions on siliconized glass. After incubation, the nonadherent cells were discarded and the adherent cells were removed by a 30 min incubation with 37°C EDTA, followed by gentle scraping with a rubber policeman. The nonadherent cells were PC that did not adhere to plastic after 1 h incubation at standard conditions. The PC were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF

monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice with the other test groups.

easily removed, it was determined that the lidocaine was toxic to the PC and MEF monolayers (data not shown).

Inhibition of EMCV replication with nylon wool adherent PC from mycobacteria-sensitized mice. The incubaction of lymphocytes on nylon wool columns is a generally accepted method for the separation of T lymphocytes from other cells (21,51). Primarily B lymphocytes and macrophages adhere to the wool, whereas T lymphocytes are nonadherent and elute from the column.

The results in Table 4 show that the adherent cells which were teased free of the nylon wool were markedly enriched for antiviral activity. The adherent cells inhibited replication by 2.6 $\log_{10}$ (>99%), as compared to 1.1 $\log_{10}$ inhibition with untreated PC from the same mycobacteria-sensitized mice. Furthermore, the nonadherent cells (T cells) did not inhibit EMCV replication. Of the total cells placed on the nylon wool, 7% were recovered as nonadherent, while 29% were recovered after teasing the wool. The nonadherent population was 4% phagocytic and <1% were esterase positive, whereas, the adherent cells were 37% and 12%, phagocytic and esterase positive, respectively. The unseparated-sensitized PC were 56% phagocytic and 13% esterase positive.

Inhibition of EMCV replication with PC from mycobacteria-sensitized nude mice. To more fully substantiate the unimportance of the Tcell in the nonspecific inhibition of EMCV replication, PC from C57BL/10ScN, athymic (nu/nu), and BALB/c (nu/+) mycobacteria-sensitized mice were tested (Table 5). In each mouse strain the nonviable mycobacteria

TABLE 4. Inhibition of EMCV replication with nylon wool adherent PC from mycobacteria-sensitized mice[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	5.8	1.1
Nylon wool adherent	4.3	2.6
Nylon wool nonadherent	7.0	none
Unsensitized mice		
Unseparated	6.9	none
None (media only)	7.2	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were incubated on a nylon wool column. Nonadherent cells were eluted from the column and adherent cells were teased free of the wool (see Materials and Methods). The PC were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice with the other test groups.

TABLE 5. Inhibition of EMCV replication with PC from mycobacteria-sensitized athymic (nu/nu), BALB/c (nu/+) and C57BL/10ScN mice[a]

Mice	Ratio of PC to MEF cells	
	20	2
nu/nu sensitized	3.8 (2.5)[b]	5.2 (1.9)
nu/nu unsensitized	6.3	7.1
nu/+ sensitized	5.2 (0.9)	7.0 (0.0)
nu/+ unsensitized	6.1	7.0
C57BL/10ScN sensitized	5.5 (1.6)	6.7 (0.6)
C57BL/10ScN unsensitized	7.1	7.3

[a]PC were removed from C57BL/10ScN, BALB/c (nu/+) and athymic (nu/nu) mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. The PC were incubated at PC/MEF ratios of 20 or 2 on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Numbers in parentheses indicate the $\log_{10}$ inhibition of EMCV replication as determined by comparing virus titers of cultures incubated with similar ratios of PC from unsensitized and mycobacteria-sensitized

mice of the same strain.

elicited PC that inhibited EMCV replication. Interestingly, PC from the athymic (nu/nu), haired littermates (nu/+), and C57BL/10ScN mice inhibited EMCV replication 2.5, 0.9, and 1.6 $\log_{10}$, respectively. Of significance is the data which indicates that PC from athymic mice were much more effective than the other two mouse strains when used at a PC/MEF ratio of only 2. At this lower ratio, PC from mycobacteria-sensitized nude mice inhibited replication 1.9 $\log_{10}$, or approximately 99%, whereas PC from the C57BL/10ScN mice resulted in minimal inhibition, and PC from the littermates were ineffective (Table 5).

Inhibition of EMCV with Hypaque-density gradient separated PC from mycobacteria-sensitized mice. The data at this point appeared to indicate that the effector cell was adherent and not a functionally mature T lymphocyte. Because high concentrations of neutrophils were observed in the PC, procedures were used to enrich for this cell. The data in Table 6 indicate that the neutrophil-rich pellet of a Hypaque-density gradient, which contained 52% neutrophils and 7% esterase positive cells, was ineffective at inhibiting EMCV replication (0.4 $\log_{10}$ inhibition). In contrast, the interface fraction of the gradient which contained only 10% neutrophils and 32% esterase positive cells inhibited viral replication by 1.6 $\log_{10}$, as compared to 1.4 $\log_{10}$ for the unseparated cells. In experiments of this type, 10% of the cells added to the gradient were recovered at the interface and 11% were present in the pellet.

Similar results were detected with Hypaque-density gradient separated PC from mycobacteria-sensitized athymic mice. The data in Table 7 show that unseparated PC from sensitized nude mice inhibited EMCV

TABLE 6. Inhibition of EMCV replication with Hypaque-density gradient separated PC from mycobacteria-sensitized mice[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	5.2	1.4
Interface	5.0	1.6
Pellet	6.2	0.4
Unsensitized mice		
Unseparated	6.6	none
None (media only)	7.0	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 μg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were separated on a Hypaque-density gradient (see Methods and Materials). The PC were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice

with the other test groups.

TABLE 7. Inhibition of EMCV replication with Hypaque-density gradient separated PC from mycobacteria-sensitized athymic (nude) mice[a]

Origin of PC	Viral titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	5.4	1.3
Interface	5.0	1.7
Pellet	6.1	0.6
Unsensitized mice		
Unseparated	6.7	none
None (media only)	6.7	none

[a]PC were removed from nude mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were separated in a Hypaque-density gradient (see Materials and Methods). The PC were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice

with the other test groups.

1.3 $\log_{10}$, whereas cells at the interface of the gradient inhibited replication 1.7 $\log_{10}$ (>90%). This was an enrichment process in that cells in the pellet inhibited virus replication by only 0.6 $\log_{10}$. In this experiment, no neutrophils and only 6% esterase positive cells were present at the interface. In contrast, 41% of the cells in the pellet were neutrophils and 2% were esterase positive.

The Hypaque-density gradient-enriched PC from mycobacteria-sensitized nude mice also were cultured to determine if they released an antiviral mediator. It can be seen in Table 8 that a mediator was present in the supernatant fluids of the unseparated PC from sensitized mice and that this activity was markedly enhanced in supernatant fluids prepared from cells present at the interface (1.3 vs. 3.8 $\log_{10}$ inhibition). Activity similar to that detected with the unseparated PC was present in supernatant fluids prepared from cells in the pellet. Supernatant fluids removed from PC cultures of unsensitized mice did not contain antiviral activity.

Effect of nonphagocytic PC from mycobacteria-sensitized mice on EMCV replication. The effector cell from mycobacteria-sensitized mice responsible for inhibition of EMCV replication has had thus far been shown to be adherent to several surfaces, but not a functionally mature T cell nor a neutrophil. The next characterization experiment involved treatment of PC with carbonyl iron and removal of the phagocytic cells with magnetic force. The carbonyl iron was coated with protein because nonphagocytic cells can adhere to untreated carbonyl iron and there by distort the separation procedure (44).

TABLE 8. Inhibition of EMCV replication with supernatant fluids prepared from cultures of Hypaque-density gradient separated PC from mycobacteria-sensitized nude mice[a]

Origin of supernatant fluid	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized PC		
Unseparated	5.7	1.3
Interface	3.2	3.8
Pellet	5.9	1.1
Unsensitized PC		
Unseparated	7.0	none
None (media only)	7.0	none

[a]PC were removed from athymic (nu/nu) mice that were unsensitized or that had been sensitized i.p. 3 wk previously sensitized with 500 µg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were separated on a Hypaque-density gradient and subsequently incubated for 24 h in plastic wells at 4.0×10^6 PC/ml. The supernatant fluids were collected, centrifuged and incubated on EMCV-infected monolayers to determine if they contained antiviral activity (see Materials and Methods).

[b]Inhibition of EMCV replication was determined by comparing virus

titers of cultures incubated with supernatant fluids from unseparated PC cultures of unsensitized mice with the other test groups.

The data in Table 9 indicate that the effector cell did not phagocytose carbonyl iron. The nonphagocytic cells, which consisted of 12% of the total cells, inhibited EMCV replication by 1.3 $\log_{10}$; the unseparated PC from the same mycobacteria-sensitized mice inhibited replication by 1.2 $\log_{10}$. Evidence that phagocytic cells had been depleted by this treatment was shown with the latex phagocytosis studies and esterase stain; 3% of the nonphagocytic cells were latex positive and 2% were esterase positive, whereas in contrast, the unseparated PC from mycobacteria-sensitized mice consisted of 61% and 39% phagocytic and esterase positive cells, respectively. Since the nonphagocytic cells inhibited EMCV replication, it was meaningful to investigate the inhibitory ability of the phagocytic cells associated with the iron. This was attempted but was unsuccessful because the iron associated with the cells was cytotoxic to the MEF monolayers. Cells from the carbonyl iron experiments also were cultured to determine if they released an antiviral mediator. The results in Table 10 show that supernatant fluids prepared from cultures of unseparated PC of mycobacteria-sensitized mice inhibited viral replication 2.3 $\log_{10}$ (>99%). Interestingly, the nonphagocytic cells which previously had been shown to inhibit EMCV replication (Table 9), also released an antiviral inhibitor (1.5 $\log_{10}$ inhibition).

Hypaque-density gradients and removal of phagocytic cells with protein-coated carbonyl iron have been shown to enrich for effector cell(s) present in PC of mycobacteria-sensitized mice (Tables 8,9). The question thus arose as to whether these techniques had enriched for a similar subpopulation of effector cells. The data in Table 11 show the

TABLE 9. Effect of nonphagocytic PC from mycobacteria-sensitized mice on EMCV replication[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	5.0	1.2
Nonphagocytic	4.9	1.3
Unsensitized mice		
Unseparated	6.2	none
None (media only)	6.8	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the cells were incubated twice for 45 min with protein-coated carbonyl iron at standard conditions. After each treatment, cells which had phagocytosed iron were drawn off with magnetic force. The PC were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice with the other test groups.

TABLE 10. Inhibition of EMCV replication with supernatant fluids prepared from cultures of nonphagocytic cells from mycobacteria-sensitized mice[a]

Origin of supernatant fluids	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-senstized PC		
Unseparated	4.7	2.3
Nonphagocytic	5.5	1.5
Unsensitized PC		
Unseparated	7.0	none
None (media only)	7.3	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were incubated twice for 45 min with protein-coated carbonyl iron. After each treatment the cells that had phagocytosed iron were drawn off with magnetic force. The nonphagocytic cells then were incubated for 24 h in plastic wells at 4.0×10^6 PC/ml. The supernatant fluids were collected, centrifuged and then incubated on EMCV-infected MEF monolayers to determine if they contained antiviral activity (see Materials and Methods).

[b]Inhibition of EMCV replication was determined by comparing virus

titers of cultures incubated with supernatant fluids from unseparated PC cultures of unsensitized mice with the other test groups.

TABLE 11. Inhibition of EMCV replication with PC from mycobacteria-sensitized mice that had been enriched by Hypaque-density gradients and phagocytosis of protein-coated carbonyl iron[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	4.3	1.4
Interface of Hypaque-density gradient	3.5	2.2
Nonphagocytic from the interface of Hypaque-density gradient	3.9	1.8
Unsensitized mice		
Unseparated	5.7	none
None (media only)	6.2	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the mycobacteria-sensitized PC were separated on a Hypaque-density gradient. The cells at the interface of the gradient then were treated twice with protein-coated carbonyl iron and magnetic force (see Materials and Methods). The PC were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were

harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice with the other test groups.

results of a typical experiment in which two enrichment techniques were combined. It can be seen that unseparated PC from mycobacteria-sensitized mice inhibited replication 1.4 $\log_{10}$ (>90% inhibition), and that the inhibitory activity was enhanced with cells present at the interface of the Hypaque-density gradient (2.2 $\log_{10}$ inhibition; >99%). Furthermore, when cells at the interface were treated with carbonyl iron, the remaining nonphagocytic cells inhibited EMCV replication to essentially the same degree as the untreated cells at the interface.

Inhibition of EMCV replication with PC from mycobacteria-sensitized mice that had been incubated with protein-coated silica. To further substantiate that a macrophage was not the effector cell, studies were undertaken to determine the effect that phagocytosis of silica, a purported macrophage toxin (43), had on the inhibition of viral replication by PC. Initially, various concentrations and intervals of exposure to uncoated silica were used and it was found that silica killed nearly all of the PC by 24 h. Coating of the silica with protein, however, prevented this indiscriminate killing (data not shown).

The data in Table 12 show that incubation of PC with silica for 24 h did not abrogate the ability of PC to inhibit EMCV replication; the silica-treated PC and the untreated controls inhibited replication to a similar degree (>90%). Evidence that silica treatment had eliminated macrophages was subjective because viability counts with trypan blue exclusion were difficult to decipher; the silica ladened cells were opaque and very similar in appearance to the darkly-stained nonviable cells. Indirect evidence that the silica was toxic was shown in the

TABLE 12. Inhibition of EMCV replication with PC from mycobacteria-sensitized mice that have been incubated with protein-coated silica[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
Unseparated	5.2	1.1
24 h silica-treated	5.1	1.2
24 h control	5.1	1.2
Unsensitized mice		
Unseparated	6.3	none
None (media only)	6.8	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. The mycobacteria-sensitized PC were incubated either with or without protein-coated silica for 24 h. After incubation the PC were washed twice and incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with 2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unseparated PC from unsensitized mice

with the other test groups.

total number of cells that were recovered after silica treatment; twice as many cells were recovered from the tubes in which cells were incubated without silica.

Inhibition of EMCV replication by irradiated PC from mycobacteria-sensitized and normal mice. Experiments were done to determine if the effector cell was sensitive to irradiation. The data in Table 13 indicate that PC treated with 2000 r were half as effective, and that those treated with 5000 r were essentially ineffective in the inhibition of EMCV replication as compared to the untreated controls (1.3 $\log_{10}$ inhibition). These same irradiated cells also were cultured to determine if they released antiviral mediator(s). The results demonstrate that, in contrast to the inability of PC to inhibit EMCV replication with increasing levels of irradiation, supernatant fluids from these cells had an enhanced inhibitory activity; inhibition of viral replication increased from 1.1 $\log_{10}$ with supernatant fluids prepared from untreated cells, to 1.7 $\log_{10}$ and 2.2 $\log_{10}$ with supernatant fluids prepared from PC that were administered 2000 r and 5000 r, respectively (Table 14).

This discordant data prompted a study to determine if supernatant fluids harvested from irradiated PC cultures after 2, and 25 h of incubation contained antiviral components that may have been released from the damaged cells. The results (Table 15) indicate that a virucidal factor was released from irradiated PC; EMCV replication was not affected by supernatant fluids produced from unirradiated PC, whereas, supernatant fluids prepared from PC that had received 2000 r and 5000 r contained a factor(s) which decreased the number of infectious viral

TABLE 13. Effect of irradiated PC from mycobacteria-sensitized and unsensitized mice on EMCV replication[a]

Origin of PC	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized mice		
No treatment	4.7	1.3
2000 r	5.0	0.7
5000 r	5.5	0.5
Unsensitized mice		
No treatment	6.0	none
2000 r	5.7	none
5000 r	6.0	none
None (media only)	6.4	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the PC were administered 2000 r, 5000 r or were used untreated (see Materials and Methods). After treatment, the PC were washed twice and then were incubated at a PC/MEF ratio of 20 (6.0×10^5 PC/well) on MEF monolayers that had been infected for 2 h with

2-4 PFU of EMCV. 18 h later entire cultures were harvested and titrated for EMCV.

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with unsensitized and mycobacteria-sensitized PC that received similar treatments.

TABLE 14. Inhibition of EMCV replication with supernatant fluids prepared from irradiated PC from mycobacteria-sensitized and unsensitized mice[a]

Origin of supernatant fluids	Virus titer (PFU/0.2 ml, $\log_{10}$)	Inhibition of viral replication ($\log_{10}$)[b]
Mycobacteria-sensitized PC		
No treatment	5.2	1.1
2000 r	4.8	1.7
5000 r	4.5	2.2
Unsensitized PC		
No treatment	6.3	none
2000 r	6.5	none
5000 r	6.2	none
None (media only)	6.8	none

[a]PC were removed from C57BL/10ScN mice that were unsensitized or that had been sensitized i.p. 3 wk previously with 500 µg of nonviable M. tuberculosis. Portions of the PC were administered 2000 r, 5000 r or were used untreated (see Materials and Methods). After treatment, PC were washed twice and then were incubated for 24 h in plastic wells at

$4.0x10^6$ PC/ml. The supernatant fluids were harvested, centrifuged and then incubated on EMCV-infected MEF monolayers to determine if they contained antiviral activity (see Materials and Methods).

[b]Inhibition of EMCV replication was determined by comparing virus titers of cultures incubated with supernatant fluids from cultures of unsensitized and mycobacteria-sensitized PC that had received similar treatments.

TABLE 15. Effect of supernatant fluids prepared from irradiated mycobacteria-sensitized PC on EMCV replication[a]

Origin of supernatant fluids	Interval PC incubated to produce supernatant fluids	
	2h	25h
	(average PFU of 2 wells)	
Mycobacteria-sensitized PC		
Unsensitized	17	17
2000 r	20	12
5000 r	19	11
None (media only)	17	

[a]PC were removed from C57BL/10ScN mice that had been sensitized with 500 µg of nonviable M. tuberculosis. Portions of the PC were administered 2000 r, 5000 r or were untreated; after treatment, PC were washed twice and then incubated at 4.0×10^6 PC/ml. Separate samples were harvested at 2 and 25 h. The supernatant fluids harvested were centrifuged and then incubated for 1 h at 37°C with EMCV (20 PFU/0.2 ml). The virus-containing supernatant fluids then were overlayed on ML monolayers to determine plaque counts (see Materials and Methods). Inactivation of the virus was determined by comparing the average number of plaques/monolayer.

particles. This factor, possibly enzymatic in nature, appears to explain the enhanced antiviral activity in supernatant fluids prepared from irradiated cells.

DISCUSSION

Mice usually die from EMCV infection as a result of CNS disease (20). In an *in vivo* model of nonspecific resistance used by Lodmell and Ewalt, female C57BL/10ScN mice sensitized either i.v. or i.p. with nonviable *Mycobacteria tuberculosis* suspended in an oil-in-saline emulsion were markedly protected aginst an i.v., i.p., s.c. or i.m. lethal challenge of EMCV (30). Studies to determine the mechanism(s) by which the nonviable *M. tuberculosis* protected mice against EMCV showed that interferon was not detectable in the sera of mycobacteria-sensitized mice, and that EMCV neutralizing antibody was present in <50% of the mice which had survived EMCV challenge (30). The nonviable mycobacteria emulsion also protected athymic nude mice against EMCV (31). Interestingly, the immunosuppressive drug, cyclophosphamide, and the purported macrophage toxin, silica, when administered to mycobacteria-sensitized euthymic mice abrogated protection against EMCV (31). To further elucidate this mechanism of nonspecific protection, an *in vitro* model was developed in which peritoneal cells from mycobacteria-sensitized mice were used to inhibit EMCV replication in MEF monolayers (Pusateri, *et al.*, manuscript accepted for publication).

The study described herein was done to determine the effector cell(s) of the PC which was involved in nonspecific resistance. PC were separated by various techniques to purify cell populations used in the *in vitro* viral inhibition assay. Evidence that silica abrogated *in vivo* resistance established by the nonviable mycobacteria sensitization prompted initial attention on the macrophage as the effector cell. Since it is known that macrophages are adherent (39), techniques were

used to separate cells by their facility to adhere to various surfaces. While testing various surfaces for cellular adherence it was recognized that yields from the cell separation techniques were quite low. This necessitated the miniturization of the viral inhibition assay from monolayers grown in 20 mm^2 tissue culture wells to 2.8 mm^2 wells so that the appropriate PC/MEF ratio could be used. Experiments performed with this miniturized system were successful (>99% inhibition of viral replication; Table 1), and all subsequent viral inhibition assays were performed on monolayers in 2.8 mm^2 wells.

Adherence of PC to plastic flasks resulted in an excellent separation of adherent and nonadherent cell populations. Nonetheless, the adherent cells could not be used in the inhibition assay because they were so tenaciously attached they could not be recovered as viable cells. To circumvent this technical problem, baby hamster kidney microexudate-coated plastic flasks, as described by Ackerman and Douglas (1), were used for attachment and recovery of high concentrations of viable adherent cells. It was determined that the adherent cells were more effective at inhibiting EMCV replication than the nonadherent cells from uncoated plastic flasks (2.6 $\log_{10}$, or >99% inhibition; Table 2). These results were confirmed by the recovery of high concentrations of viable adherent PC from siliconized glass flasks (2.2 $\log_{10}$, >99% inhibition; Table 3). The similar results from these two separation procedures indicated that the effector cell which inhibited EMCV replication was adherent.

In an attempt to identify the effector cell, Wright's stain initially was employed; cells stained in this manner were found to be

inadequately differentiated. Classic cell types were readily recognized, but many cells were of intermediate morphology and cell differentiation became rather subjective. Because of this ambiguity, Wright's stain was only used to differentiate neutrophils. For further identification, PC were tested for their ability to phagocytize latex spheres. Scoring phagocytic cells was generally straightforward. On occassion, however, it was difficult to determine if the latex was intracellular or extracellular.

Use of the Wright's stain and latex ingestion techniques for cell differentiation indicated that both BHK microexudate and siliconized glass adherent cells were similar in their neutrophil content as compared to the unseparated population, whereas, the number of phagocytes had increased. This evidence suggested a need for an additional and more specific criteria by which to identify macrophages. This was accomplished by latex ingestion and staining of PC for nonspecific esterase as described by Koski, et al. (28). The nonspecific esterase stain is specific for macrophages, but there is one report in which another cell has been described to be esterase positive (37). This stain was, however, in combination with the latex phagocytosis studies a more accurate method of identifying macrophages than the Wright's stain.

Attempts also were made to separate adherent and nonadherent PC on surfaces such as waxpaper, paraffin, untreated glass, cellophane, teflon and aluminum foil but none gave better results than those attained with BHK microexudate and siliconized glass. In addition, the anesthetic lidocaine, was used to loosen adherent PC as described by Raff, et al. (47). Various incubation intervals and concentrations of lidocaine were

tested with PC. It was determined that low concentrations of lidocaine did not loosen cells well enough to recover sufficient amounts of viable PC, whereas higher concentrations were toxic to the PC. Furthermore, MEF monolayers were sensitive to the lidocaine in that viral titers in MEF monolayers were reduced if the PC were not thoroughly washed.

During the initial cell separation procedures it became apparent that particular cell populations were present in PC of untreated and mycobacteria-sensitized mice. Monocytes, neutrophils and lymphocytes were present in PC, but other cell types were infrequently seen; eosinophils were rarely, if ever detected and basophils were <1% of the PC population of normal mice and rarely present in PC from sensitized mice. It seemed reasonable therefore, that such sporatically occurring cells would not be the effector cell, particularly since eosinophils and basophils have been cited as effectors in allergic reactions (6), and are not known to be associated with antiviral immunity. Furthermore, these cells have been described as poorly phagocytic (nonadherent) (6), results which do not conform to the adherent properties of the effector cell in the present investigation.

Neutrophils have been considered to be primarily involved with antibacterial rather than antiviral host defenses (49). Recently, however, these cells have been cited as effectors against viral infections (50). Because substantial concentrations of neutrophils were observed in PC from mycobacteria-sensitized mice, the neutrophils were separated on a Hypaque-density gradient and tested for inhibition of virus replication. Cell populations that were 50% neutrophils did not inhibit EMCV replication, whereas, the remaining cells which contained few

neutrophils were markedly effective (1.6 $\log_{10}$ inhibition; Table 6). Thus neutrophils did not appear to be associated with the viral inhibition expressed by PC from mycobacteria-sensitized mice.

A mechanism of protection associated with tumor and virus-infected cell systems is cytolysis. A variety of cells have been cited for their involvement in cytolysis including killer cells (53), null cells (54), macrophages (48,52), natural killer cells (38), and T lymphocytes (25). Because none of these cells is adherent, except the macrophage, their involvement in the inhibition of EMCV replication would appear to be suspect because the effector cell has been shown to adhere to various surfaces. Furthermore, cytolysis is not involved in the in vitro inhibition of EMCV replication in this model system (Pusateri, et al. manuscript accepted for publication). Thus, the possibility that the macrophage may act as a lytic cell in this system has been discounted, but one of its many other features may still have been of importance.

Inhibition of viral replication by PC from mycobacteria-sensitized mice did not appear to involve the T lymphocytes because the in vivo protection of mycobacteria-sensitized mice was shown to be thymus-independent (31); neonatally-thymectomized and athymic nude mice were protected against a lethal EMCV challenge by the mycobacteria. To confirm the in vivo results, PC were harvested from mycobacteria-sensitized mice and filtered through a nylon wool column; two populations with significantly different characteristics were isolated. The purified T lymphocytes, which passed through the nylon wool column (51) were ineffective in the inhibition of replication, whereas, those cells which adhered to the nylon wool and were teased free inhibited

EMCV replication >99% (Table 4). Furthermore, PC harvested from mycobacteria-sensitized athymic nude mice were markedly effective in the inhibition of EMCV replication. Interestingly, the nude mouse PC were more effective than PC from C57BL/10ScN mice in that they inhibited replication by approximately 99% (Table 5) at a PC/MEF cell ratio of only 2; PC from conventional euthymic mycobacteria-sensitized mice were ineffective at this ratio. Thus, it appeared from the nylon wool and nude mouse studies that the functionally mature T lymphocyte was not important in the inhibition of EMCV replication.

The effector cell's ability to adhere to various surfaces and nylon wool, as well as the latex phagocytosis studies and nonspecific esterase stains strongly suggested the importance of the macrophage in the inhibition of EMCV replication. To further elucidate this possibility, macrophages were removed from the PC by phagocytosis of protein-coated carbonyl iron. Surprisingly, the remaining nonphagocytic cells which were <1% esterase positive and only 4% phagocytic markedly inhibited EMCV replication (1.3 $\log_{10}$, >90% inhibition; Table 9) and exceeded the inhibition of that attained by the unseparated PC. Irradiation of the PC from mycobacteria-sensitized mice further substantiated that the macrophage was not the effector cell since macrophages are known to be resistant to irradiation, whereas dividing cells are not (62). The amount of viral inhibition decreased significantly after the PC were irradiated with 5000 r (Table 3). Surprisingly, in discordance with the lack of inhibition attained with irradiated PC, supernatant fluids from these cells markedly inhibited EMCV replication (Table 14). To resolve these dissimilar results, supernatant fluids from the irradiated

cells were incubated with EMCV to determine if they inactivated virus. The results showed that EMCV was inactivated by a factor(s) released from the irradiated cells, and the activity of the factor correlated directly with the irradiation dose and length of time the cells were incubated in culture after irradiation (Table15). The above data suggests that the effector cell was radiosensitive even though the effect of the irradiation may have been partially masked by the factor, possibly enzymatic in nature, which was released from the irradiated cells.

Data from the phagocytosis studies with protein-coated silica also indicated that the macrophage was not directly involved in the inhibition of EMCV replication; PC which were treated for 24 h with silica were as effective as the untreated cells (1.2 $\log_{10}$, >90% inhibition; Table 12). These results are in contrast to the previous *in vivo* studies in which the i.p. injection of uncoated silica ablated resistance to EMCV. The use of uncoated silica *in vitro* resulted in poor results in that nearly all the PC were killed after 24 h of incubation. The protein restricts the spectrum of killing to only those cells which can phagocytize the silica (43). When ingested, the silica particle is stripped of its protein coat by proteolytic enzymes, and the silicic acid damages the lysosomal membranes and liberates lysosomal enzymes into the cytoplasm (2). If the silica is uncoated, silicic acid is free to destroy virtually any exposed cell membrane. Had protein-coated silica been used with the *in vivo* studies instead of untreated silica, results similar to the *in vitro* experiments may have occurred.

In view of the above results, it appears that the effector cell responsible for inhibiting EMCV replication may be the B lymphocyte.

Typically, the B cell is recognized for its role in antibody production. Nonetheless, antibodies did not appear to be involved in this model of nonspecific resistance because the mycobacteria-sensitized mice had never been exposed to EMCV antigen before the *in vitro* viral inhibition assay and supernatant fluids produced from cultures of mycobacteria-sensitized PC which inhibited EMCV replication did not neutralize EMCV (A.M. Pusateri, M.S. Thesis, 1979, Dept. of Microbiology, Univ. of Montana).

The adherent cell population of nylon wool columns has been shown to be enriched for B lymphocytes, whereas, the nonadherent population is depleted of similar cells (21). This evidence would suggest that the B cell may also adhere to other materials, which supports the finding of the present study in that the effector cell was adherent to various surfaces including nylon wool. The involvement of the B cell in the inhibition of EMCV replication also was reflected in the *in vivo* studies in this model system in that it was determined that administration of the immunosuppressive drug, cyclophosphamide, which destroys rapidly dividing cells like the B cell (61), ablated resistance of mycobacteria-sensitized mice to challenge with EMCV (31). Furthermore, it also has been shown by Handwerger and Schwartz (21) that nylon wool adherent populations from spleens were depleted of macrophages. In the present study, a marked decrease in the number of macrophages was detected in the nylon wool adherent cells as compared to the unseparated cell population. This data, in combination with that of Handwerger and Schwartz, further demonstrates that the effector cell probably was not the macrophage, and emphasizes the importance of the B cell: the nylon wool adherent population inhibited EMCV replication more effectively than the unseparated

PC from mycobacteria-sensitized mice.

Work in the present model system of nonspecific resistance to EMCV by Pusateri et al. (manuscript accepted for publication) has shown that PC from mycobacteria-sensitized mice secreted a soluble factor that inhibited EMCV replication. This mediator had the characteristics of an immune type II interferon. It is interesting to note that B cells also have been shown to produce type II interferon in response to a variety of stimuli including viruses (15), mitogens (14), and bacteria (23). In a related model, splenocytes from mice injected i.p. with heat-killed Corynebacterium parvum produced a type II interferon (23). Nylon wool columns and plastic surfaces removed the interferon-producing cells. Even though T lymphocytes are known to produce immune type II interferon (14), they were not necessary in this study because spleen cells treated with anti-θ and complement, in addition to splenocytes from C. parvum-treated nude mice were effective. It was suggested, as a result of these cell separation studies, that interferon was produced by B lymphocytes, but that macrophage cooperation was required (23). Other studies also have shown that macrophage-lymphocyte cooperation is necessary for the production of interferon (13,14,15).

In whatever capacity macrophages participate with B lymphocytes to produce interferon, their cooperation in this investigation did not appear to be prolonged after establishment of in vitro cultures. The B cell appeared to produce interferon in culture without the macrophage for a relatively short period. After this time the macrophage was needed for continued interferon production. Evidence for this was shown in the activity of supernatant fluids produced from unseparated and separated

cell populations. Supernatant fluids prepared from nonphagocytic cells which remained after treatment with carbonyl iron were nearly 1 $\log_{10}$ less efficacious in the inhibition of EMCV replication than supernatant fluids produced from unseparated PC from the same mycobacteria-sensitized mice. Furthermore, supernatant fluids prepared from PC of mycobacteria-sensitized nude mice that had been separated on a Hypaque-density gradient were approximately twice as effective as the unseparated nude PC (Table 8). Interestingly, the cells at the interface contained a much higher concentration of macrophages than did the unseparated cells. Thus, cell populations depleted of macrophages produce less effective supernatant fluids and cell populations which contain higher concentrations of macrophages for presumably, more efficient cell cooperation, produce highly active supernatant fluids.

In this study, the effector B cell was identified by indirect procedures. This investigation could continue by identifying the B cell with more specific techniques. Additional studies also could include 1) the duration of B cell interferon production in vitro after macrophage priming 2) the nature of the communication between the B cell and macrophage that is required for interferon production (contact or lymphokine) and 3) the effect of normal vs. sensitized macrophages on interferon production by the sensitized B cells.

It also would be enlightening to return to the in vivo model and recheck some of the initial observations. Since type II interferon produced by mycobacteria-sensitized PC inhibited EMCV replication in vitro, perhaps interferon also could be detected by more sensitive techniques in the mycobacteria-sensitized mice. Modification of the

original *in vivo* silica treatment by coating silica with protein may confirm the findings of the *in vitro* study. Ultimately, with refined isolation techniques and better cell identification, selected cell populations from mycobacteria-sensitized mice could be transferred into normal animals which then would be challenged with EMCV to determine if the same effector cells that inhibited viral replication *in vitro* also protected mice *in vivo*.

In summary, PC harvested from C57BL/10ScN and athymic (nu/nu) mice sensitized i.p. with nonviable *Mycobacteria tuberculosis* nonspecifically inhibited EMCV replication *in vitro*. The effector cell responsible for this inhibition was adherent, nonphagocytic, radiosensitive, nonesterase positive, and neither a neutrophil nor a functionally mature T lymphocyte. Furthermore, it could be enriched for in Hypaque-density gradients, with a protein-coated carbonyl iron treatment or by adherence to BHK microexudate, siliconized glass and nylon wool. The results suggest that the effector cell is a B lymphocyte and that its effect on viral replication is expressed by secretion of type II interferon. The interferon was released from cell populations that did not contain T lymphocytes or macrophages, but was enhanced in the presence of macrophages.

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