Association of Persistent Synthesis of Viral DNA with Macrophage Accessory Cell Dysfunction Induced by Avian Retrovirus Myeloblastosis-associated Virus of Subgroup B Inducing Osteopetrosis in Chickens

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ABSTRACT

This investigation concentrates on a regenerative anemia and immunosuppression occurring in the absence of osteopetrosis. Polyclonal activation of T-cells was used as an in vitro test system to study immunosuppression induced by the avian myeloblastosis-associated virus of Subgroup B inducing osteopetrosis [MAV-2(O)]. T-cell unresponsiveness in vitro was attributed to a defect in an accessory cell function of the macrophage. Counterflow centrifugation fractionation followed by mixing experiments indicated that the T-cell population from immunosuppressed chickens responded to mitogen stimulation when added to control macrophage cultures. In addition, lymphocyte fractions from uninfected chickens were unresponsive when added to macrophage cultures isolated from MAV-2(O)-infected chickens. Cultured splenic macrophages isolated from infected chickens contained high levels of both integrated and unintegrated viral DNA and formed syncytia by 21 days in culture. The macrophages remained viable and exhibited mature functional characteristics during mitogen stimulation assays. Therefore, it was speculated that the persistent synthesis of retrovirus DNA might be involved in the inability of infected macrophages to function as accessory cells.

INTRODUCTION

Animals infected with oncogenic viruses frequently develop a compromised immune system (1). Infection of chickens with acute leukemia viruses (myelocytomatosis virus MC-29, AEV, AMV, and REV) results in suppression of mitogen-induced proliferation of T-cells (2). It is now clear from these investigations that the immunosuppression is associated with helper viruses present in stocks of the acutely oncogenic components. Among these helper viruses is the myeloblastosis-associated virus MAV-2(O), which is an end point-purified isolate from an AMV stock (3). Injection of MAV-2(O) into 10-day-old chick embryos leads to the appearance of osteopetrosis within the first week after hatch, and a 100% incidence by 2 to 3 wk of age (4). Osteopetrosis is a disease in which osteoblasts undergo abnormal growth and differentiation, leading to the accumulation of excessive bone (5, 6). Osteopetrotic chickens develop progressive anemia and immunosuppression during the development of massive bone lesions (7, 8). Suppression of humoral and cellular immune functions during bone lesion development is accompanied by a marked atrophy of the lymphoid organs. These disorders appear in part to be the indirect result of a progressive reduction of the hematopoietic tissue as rapidly proliferating osteoblasts occlude the marrow cavity.

Therefore, the conditions are chronic and occur up until the death of the animal. In contrast, chickens infected 10 days after hatching develop a severe regenerative anemia and immunosuppression in the absence of osteopetrosis (9, 10). MAV-2(O)-induced anemia and immunosuppression occurring in the absence of bone lesion formation are transient, and neutralizing antibodies appear during convalescence (9). Polyclonal activation of T-cells with mitogen has been used as an in vitro test system to study immunosuppression induced by MAV-2(O) (8, 10, 22). An in vitro blastogenic response to Con A is T-cell specific in the chicken (11, 12), and T-cell blastogenic response to mitogen correlates with the ability of birds to elicit a cellular immune response in vivo (13). Previous studies in this laboratory have indirectly attributed splenic T-cell unresponsiveness during MAV-2(O) infection to a defect in an accessory cell function of the macrophage (10). The same studies show that blastogenic failure is not due to a lack of Con A receptor sites nor the presence of suppressor cells. In this study, we provide direct evidence for a macrophage accessory cell dysfunction and have examined defective cultured macrophages for the presence of viral-specific proteins and nucleic acids along with growth characteristics in vitro.

MATERIALS AND METHODS

Virus and Virus Assays. An end point-purified derivative of avian myeloblastosis virus, designated MAV-2(O), was used. All virus stocks were derived from Plaque Isolate 32, which was plaque purified 3 times (3). Infectivity assays were performed by plaque assay (14).

Experimental Animals. Chicks of the SC line were obtained from Hyline International, Dallas Center, IA. Cells from the SC line of chickens are group-specific antigen negative, chicken-helper-factor negative and free of retrovirus expression (15). MAV-2(0), 1 × 108 plaque-forming units/chick, was administered i.v. to 10-day-old chickens. Birds were maintained in an animal isolation facility designed to prevent spread of viruses among groups of infected animals and were fed Purina Growena Chow and water ad libitum.

Preparation of Spleen Lymphocytes and Macrophages. Spleens were removed aseptically and placed in ice-cold serum-free RPMI 1640 (Sigma, St. Louis, MO). Single cell suspensions were obtained by gentle passage through a Tenbroeck tissue homogenizer. Large aggregates and tissue fragments were removed by sedimentation at 1 × g for 10 min. Following 3 washes, erythrocytes were removed by sedimentation through a Ficoll-Paque solution (Pharmacia, Inc., Piscataway, NJ). The lymphocyte band was washed 3 additional times in ice-cold HBSS (KC Biological, Lenexa, KS) buffered with 10 mM HEPES. Lymphocytes were resuspended in serum-free RPMI 1640 and used directly in stimulation assays or fractionated by counterflow centrifugation.

Macrophages were obtained from spleen lymphocytes which had been isolated by centrifugation over Ficoll-Paque. Briefly, spleen leukocytes were incubated overnight in RPMI 1640 containing 20% fetal calf serum and 10% chicken serum. To remove nonadherent cells, plates were agitated on a microtiter shaker for 20 s, and the supernatant was discarded. After this washes the adherent cells consisted of >99% macrophages as determined by Wright staining and nonspecific esterase activity.

Lymphocyte Stimulation Assay. Spleen lymphocytes were cultured in...
4 million cells/ml. The possibility that MAV-2(O) was killing cells in splenic lymphocyte cultures derived from MAV-2(O)-infected chickens exhibited a marked inability to respond to mitogens in the presence of varying concentrations of Con A (1, 2, 4, 8, and 16 µg/ml) (16). After 48 h, the cultures were labeled with 0.5 µCi/well of [3H]-thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA) for 5 h. The cells were then harvested onto glass fiber filters, and [3H]-thymidine uptake was determined by liquid scintillation spectroscopy. Stimulation was evaluated by calculating the stimulation index (cpm of stimulated cultures/cpm of unstimulated cultures). Error calculations were computed as standard error of the mean. Levels of significance (P) were calculated by Student's two tailed t test. P values refer to differences observed between normal and infected animals of the same age.

**Counterflow Centrifugation.** Spleen cell suspensions were separated from RBCs by centrifugation on Ficoll-Paque cushions, and fractionated by counterflow centrifugation using a Beckman elutriator rotor (No. JE-6B; Beckman Instruments, Palo Alto, CA), equipped with a 5-ml separation chamber in a modified Beckman J-21C centrifuge (17, 18). All separations were performed at room temperature at a rotor speed of 2000 rpm. The elutriation medium was Ca2+- and Mg2+-free HBSS containing 0.1% bovine serum albumin, 0.1% dextrose, 0.01% MgSO4, and 25 mM HEPES buffer. The flow rate was controlled by a cardiovascular pump (No. 7013; Cole Parmer, Chicago, IL). Initially, 100 ml of elutriation medium were passed through at a flow rate of 4 to 5 ml/min to allow the spleen cells to enter the separation chamber and to remove cellular debris present in the suspension. A purified lymphocyte preparation was obtained by increasing the flow rate to 7 ml/min and collecting 150 ml. A macrophage-enriched fraction was obtained by turning off the rotor and flushing remaining cells through the elutriation system.

**Fluorescent Antibody and Reverse Transcriptase Assays.** Two million splenic leukocytes recovered from Ficoll-Paque gradients were seeded into 8 chambers/fixed gasket slides (Lab-Tek; Miles Scientific, Naperville, IL) in adherence medium. To remove adherent cells, cultures were washed extensively 2 days after initiation. Macrophage cultures were maintained in adherence medium and monitored at specific time points for the presence of viral protein production. To detect viral core M, 27,000 protein, macrophage cultures were fixed in 95% ethanol/5% acetic acid for 20 min at −20°C. Fixed slides were incubated 40 min at 37°C with rabbit anti-M, 27,000 protein. The same incubation scheme was used for the secondary goat anti-rabbit IgG (Sigma, St. Louis, MO). Fluorescent-positive cells were enumerated using an Olympus BH-2 fluorescent microscope. Virus growth was monitored by assaying macrophage culture supernatants for the presence of reverse transcriptase activity (19).

**Preparation of Cellular DNA, Restriction Endonuclease Analysis, and Nucleic Acid Hybridization.** Southern analysis of HindIII-digested DNA samples was performed by hybridization to a 1300-bp probe (pBB-12) specific for the gag region of RAV-1 (15, 20). This probe detects a 3.1-kilobase DNA fragment present in MAV-2(O). A 5.1-kilobase 5′ junction fragment derived from ev-1, which also hybridizes to pBB-12, served as an internal control for sequences present at a frequency of one copy per haploid genome. The amount of integrated and unintegrated viral DNA was calculated by determining the ratio of the 3.1- and 5.1-kilobase fragments based upon densitometric scanning of autoradiographs. The densitometric reading of the 8.0-kilobase complete MAV-2(O) genome, detected in undigested samples, was subtracted from the 3.1-kilobase reading present in corresponding HindIII-digested samples in order to calculate the ratio of unintegrated to integrated DNA.

**RESULTS**

**Kinetics of the Suppressed Mitogenic Response.** T-cells present in splenic lymphocyte cultures derived from MAV-2(O)-infected chickens exhibited a marked inability to respond to Con A from 3 to 21 days postinfection (Fig. 1). Lymphocyte blastogenesis was suppressed over a cell density range of 0.5 to 4 million cells/ml. The possibility that MAV-2(O) was killing the cultured lymphocytes was evaluated by trypan blue exclusion. No difference was found in trypan blue exclusion between infected and uninfected lymphocyte populations in culture. Flow cytometric analysis using a monoclonal antibody specific for mature avian T-cells (21) did not detect any significant difference between the splenic T-cell populations of infected and uninfected chickens. T-cell-conditioned medium was titrated into cell cultures/cpm of unstimulated cultures). Error calculations were computed as standard error of the mean. Levels of significance (P) were calculated by Student’s two tailed t test. P values refer to differences observed between normal and infected animals of the same age.

**Effect of T-Cell-conditioned Medium on the Suppressed Mitogenic Response.** Recent investigations involving mitogen unresponsiveness of splenic T-cells during osteopetrosic bone lesion development have implicated a defect in the ability of the T-cell to either respond to TCGF or to synthesize TCGF (22). To determine whether a similar mechanism was involved in the transient immunosuppression occurring in the absence of osteopetrosis, T-cell-conditioned medium was titrated into unresponsive cultures. TCGF-enriched supernatants were prepared and collected as previously described (23). No significant increase in the blastogenic response was observed (Fig. 2) following addition of the growth factor-enriched medium into the unresponsive cultures.

**Stimulation of Splenic Lymphocyte and Macrophage Fractions following Counterflow Centrifugation.** A method was developed to fractionate the splenic leukocyte population into highly enriched lymphocytes and macrophages. Separation was accomplished by the use of counterflow centrifugation based on the size differences of these cell types. Flow rates were manipulated to yield two fractions, a lymphocyte fraction and a macrophage fraction (see “Materials and Methods”). The lymphocyte fraction was homogeneous as judged by morphological examination using Wright’s stain and contained less than 1% contaminating macrophages as determined by nonspecific esterase staining. The macrophage fraction contained lymphocytes, granulocytes, and other large blast-like cells to various degrees depending on the experiment. The lymphocyte fraction

![Fig. 1. Pattern of mitogen blastogenesis in spleen cells from MAV-2(O)-infected and age-matched uninfected chickens. Pooled spleen leukocytes were obtained from uninfected and infected birds. Washed cells were purified over a Ficoll-Paque cushion. Two million cells per well were stimulated with 2 µg/ml Con A. Assays were performed in triplicate. Cells were cultured in serum-free RPMI 1640 for 48 h, and 0.5 µCi of [3H]-thymidine was added during the last 5 h. Cells were harvested onto glass fiber filters, and radioactivity was measured by scintillation spectroscopy. Stimulation was evaluated by calculating the stimulation index (cpm of stimulated cultures/cpm of unstimulated cultures).](image-url)
was consistently unresponsive in mitogen stimulation assays, while the macrophage fraction responded in proportion to the amount of contaminating lymphocytes. Further purification of the macrophage fraction using plastic adherence removed contaminating nonadherent cells. Blastogenesis assays demonstrated that lymphocytes required the accessory function of macrophages during the mitogenic response to Con A (Fig. 3).

Reconstitution of the Suppressed Mitogenic Response with Normal Macrophages. Increased numbers of normal splenic macrophages enriched by counterflow centrifugation and added to unresponsive cultures significantly increased the blastogenic activity of the T-cell population (Fig. 4). Increased blastogenic activity due to macrophage division was ruled out by observing that control cultures, consisting of macrophages alone, incorporated label at levels only slightly above unstimulated lymphocyte control values. These results indirectly implicate an accessory cell dysfunction of the macrophage population in splenic leukocyte preparations obtained from MAV-2(O)-infected chickens.

Direct Evidence for a Macrophage Accessory Cell Dysfunction in Spleen Lymphocyte Cultures from MAV-2(O)-infected Chickens. Splenic lymphocyte fractions derived from both infected and uninfected chickens did not respond to Con A when combined with the purified macrophage fraction derived from the immunosuppressed chickens (Fig. 5). In contrast, splenic lymphocyte fractions derived from both infected and uninfected chickens responded normally when combined with the highly purified macrophage fraction obtained from normal uninfected chickens. These results provide direct evidence that the splenic macrophages from MAV-2(O)-infected chickens were unable to supply an accessory function to the T-cells vital for mitogenic responsiveness. The data provide further evidence that the T-cells from the nonresponsive cultures were present and fully functional when supplied with the proper accessory cell component(s).

Examination of Macrophage Cultures for Virus-specific Proteins. Splenic macrophage cultures derived from immunosuppressed chickens were examined for the presence of viral core M, 27,000 protein. Low levels of viral protein production were also observed in particular reverse transcriptase assays. Reverse transcriptase activity in supernatants removed from macrophage cultures detected a 10-fold increase above control cultures by 12 days in vitro. The amount of enzyme activity remained low over a 4-wk period relative to MAV-2(O)-infected chick embryo fibroblast cultures. Throughout this period, MAV-2(O)-infected macrophage culture titers remained 50- to 100-fold less than titers produced by infected fibroblasts of identical density. Splenic macrophages developed syncytia beginning 14 days after cultures were initiated. By 30 to 40 days in culture multinucleated giant cells dominated the infected splenic macrophages.
MYELOBLASTOSIS-ASSOCIATED VIRUS-INDUCED MACROPHAGE DYSFUNCTION

Fig. 4. Partial reconstitution of the suppressed mitogen response with normal macrophages. Pure macrophage cultures were obtained by plating the normal spleen macrophage fraction separated by counterflow centrifugation in adherence medium (see "Materials and Methods"). Two million spleen leukocytes purified by Ficoll-Paque centrifugation from uninfected and infected birds were added to the macrophage cultures and stimulated as described in Fig. 1. ■, uninfractionated splenic leukocytes from infected chickens; □, uninfractionated splenic leukocytes from uninfected controls. Bars, SD.

Fig. 5. Direct evidence for a macrophage accessory cell dysfunction in spleen lymphocyte cultures from MAV-2(O)-infected chickens. Macrophage cultures were obtained following Ficoll-Paque centrifugation of spleen leukocytes, counterflow centrifugation, and plastic adherence. Spleen macrophages isolated from uninfected (top) and 12-day postinfected (bottom) chickens were set up in flat-bottomed 96-well plates at 60,000 cells/cm². Two million purified spleen lymphocytes isolated by counterflow centrifugation were added to both macrophage cultures and stimulated as described in Fig. 1. ■, lymphocytes from infected chickens; □, control cultures. Bars, SD.

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DISCUSSION

Infection of 10-day-old chicks with MAV-2(O) rapidly reduced the ability of splenic T-cells to proliferate in response to Con A stimulation. Previous studies have shown that early suppression of the mitogenic response is a common feature of infection by nondefective helper leukemia viruses (2). Our interest was to determine the immunosuppressive mechanism used by MAV-2(O) in vitro.

Examination of the nonresponsive cultures indicated that both lymphocyte and macrophage populations were viable and present in numbers comparable to the control cultures. However, due to the lack of subset-specific monoclonal antibodies in the chicken, it cannot be ruled out that a specific lymphocyte subpopulation was missing from the unresponsive cultures.

The addition of T-cell-conditioned medium to the unresponsive cultures did not increase the blastogenic response over a wide cell density and Con A concentration range. These results indicate that MAV-2(O)-induced T-cell unresponsiveness during transient, regenerative anemia may involve a different immunosuppressive mechanism than that observed during bone tumor formation. Previous investigations involving T-cell unresponsiveness during osteopetrosis show that the addition of conditioned medium to spleen cell cultures from infected birds results in the reconstitution of the suppressed mitogenic response (22). In the same studies, T-cell mitogenic response could not be reconstituted with the addition of normal macrophages. Our results involving MAV-2(O)-induced transient immunosuppression in the absence of osteopetrosis indicate that T-cell responsiveness was significantly increased when normal spleen-derived macrophages were added to nonresponsive cultures. These results corroborate previous findings using macrophages derived from peripheral blood (10). Together these experiments provide indirect evidence for a macrophage accessory cell dysfunction. The macrophage dysfunction observed in the transient immunosuppression may represent an early stage of the severe generalized immunopathology observed to accompany osteopetrosis development in vivo (8, 22).

Splenic macrophages isolated from MAV-2(O)-infected birds were directly examined for accessory cell function using enriched lymphocyte and macrophage preparations separated by counterflow centrifugation. Neither control nor infected lymphocyte fractions responded to Con A when combined with splenic macrophage cultures isolated from MAV-2(O)-infected birds. However, both lymphocyte fractions responded at normal levels when combined with splenic macrophage cultures isolated...
from uninfected control birds. These results provide direct evidence for a defect in an accessory cell function(s) of splenic macrophages isolated from MAV-2(O)-infected chickens. In addition, it appears that the mature splenic T-cell population is present in the MAV-2(O)-infected chickens and responsive to Con A stimulation when provided with the appropriate accessory cell signal.

Direct analysis of MAV-2(O)-infected macrophages using fluorescent antibody specific for virus core M, 27,000 protein and analysis of reverse transcriptase activity detected low levels of viral protein production in vitro. In contrast, Southern blot analysis revealed high levels of both integrated and unintegrated MAV-2(O)-specific DNA. Unintegrated viral DNA is a required intermediate in the life cycle of retroviruses (24). It is detected in vitro usually only for a few days following synchronous infection of cells at high virus multiplicity (25). Unintegrated viral DNA is normally not detected in vivo, since few cells are likely to be infected immediately prior to tissue sampling. Transient production of unintegrated viral DNA following in vitro infection of chick embryo fibroblasts with certain avian retroviruses correlates directly with viral cytopathogenicity (26, 27). However, in these studies the amount of unintegrated DNA detected in killed cells was 10- to 20-fold higher than observed in the MAV-2(O)-infected macrophage cultures. In addition, previous investigations could not rule out that in vitro cytopathicity resulted from massive superinfection and membrane alteration as a result of high titer virus production (27). Low level virus production, combined with the accumulation of intermediate levels of unintegrated viral-specific DNA,
may explain the decreased cytopathic effect of MAV-2(O) on cultured splenic macrophages while interfering with specific functional properties. The results of the present investigation suggest that only a minority of macrophages from the infected animals expressed viral M, 27,000 protein. This result was consistent with the low levels of reverse transcriptase activity detected, but inconsistent with the functional paralysis observed in blastogenic assays, which suggested that the entire population of infected macrophages was impaired. The persistent synthesis of linear unintegrated MAV-2(O) DNA is not unique to the macrophage. Abnormally proliferating osteoblasts obtained during osteopetrosis, nephroblastomas, and untransformed kidney cells contain varying amounts of linear unintegrated MAV-2(O) DNA (28–30). Furthermore, linear unintegrated DNA is present in MAV-2(O)-infected chick embryo fibroblast cultures for a period exceeding that cited in the literature for other cytopathic avian leukemia viruses (27). Previous reports have detected MAV-2(O)-specific unintegrated linear DNA up to 17 days postinfection (31), and experiments in our laboratory have detected unintegrated MAV-2(O) DNA at 30 days in chick embryo fibroblast cultures. One mechanism responsible for the persistent synthesis of viral DNA is proposed to be reinfection of cells, and it has been suggested that infections that undergo the persistent synthesis of viral DNA appear to be associated with cell dysfunction (28).

Considerable new information has accumulated within the last few years on the immune systems of birds, and data show that the avian cellular immune effector mechanisms are quite similar to those of mammals (32). The role of the accessory cell in optimizing the T-cell-proliferative response to mitogens is a well-known but poorly understood phenomenon (33). The signals that accessory cells deliver appear to be multiple, involving cell-cell contact, secretion of soluble mediators, or both (34, 35). The first mechanism appears to involve binding of the mitogen by the macrophage, followed by presentation to the T-cell in a manner that induces cell activation (33). Expression of MHC Class II (Ia) antigens by the accessory cell appears to play a critical role in this presentation process (36, 37). The second mechanism by which macrophages function as accessory cells is by the elaboration of a soluble factor or factors (38). Our results suggest that the MAV-2(O)-induced macrophage dysfunction takes place at the level of macrophage-T-cell contact, because the physical presence of normal macrophages is required for blastogenic reconstitution. In addition, had the dysfunction involved the decrease of a specific soluble mediator (lymphokine or monokine), the addition of T-cell-conditioned medium should have elicited some degree of increase in T-cell blastogenesis. Our results show no significant change following the addition of T-cell-conditioned medium.

The mechanism by which the persistent synthesis of viral DNA might disrupt the accessory cell function of the infected macrophage is not known. Our results indicate the accumulation of 15.7 copies/haploid genome of unintegrated viral DNA, and 4.7 copies of integrated viral DNA after 10 days in culture. It is possible that the energy consumed by these sequences (integrated and unintegrated) and their biochemical processing interfere with antigen processing and/or presentation to the T-cell. Another possibility is that MAV-2(O)-specific proteins may occupy a process used by the macrophage which is normally utilized for the synthesis and expression of vital accessory cell receptors, such as Ia antigens. Our data do not permit an analysis of whether a few cells contain many copies of viral DNA or the entire population has the same number of copies in each cell.

The formation of syncytia, with progression to cell death, is a characteristic feature of in vitro cultures of susceptible cells infected with HIV and other lentiviruses (39). Macrophage cultures frequently contain giant multinucleated cells having more than 100 nuclei (40). In general, the HIV-induced cytopathic effect on cultured macrophages is not as profound as that in cultures of virus-infected T-cells. These similarities between MAV-2(O)-infected and HIV-infected macrophages, including persistently synthesized unintegrated viral DNA, the formation of multinucleated syncytia, and the relative resistance to the cytopathic effects of virus infection, indicate that MAV-2(O)-infected macrophages may provide a useful retrovirus model to investigate the role of mononuclear phagocytes in HIV infection.

The macrophage may play an important role in MAV-2(O) dissemination and persistence. This is of interest in the regenerative anemia and transient immunosuppression induced by MAV-2(O), since the majority of these birds exhibit a reactivation event as adults which leads to either a fatal aplastic crisis or the development of tumors, such as nephroblastomas or B-cell lymphomas.

It is now clear that macrophages are an important constituent of the hematopoietic microenvironment. Macrophages and macrophage-like cells (adventitial reticular cells) (41) have been shown to play a central role in the stimulation and regulation of both erythropoiesis and granulopoiesis (42, 43). This is especially intriguing due to the regenerative nature of the anemia and granulocytopenia which accompanies the transient accessory cell defect observed during MAV-2(O) infection.

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