Mitogen- and Viral Antigen-Induced Transformation of Lymphocytes from Normal Mink and from Mink with Progressive or Nonprogressive Aleutian Disease

SOO HWAN AN AND BRUCE N. WILKIE

Department of Veterinary Microbiology and Immunology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 1 April 1981/Accepted 28 May 1981

Peripheral blood lymphocytes (PBL) from mink with progressive Aleutian disease (AD) were shown to be significantly less responsive to phytohemagglutinin, concanavalin A, and pokeweed mitogen than were PBL from normal mink and from mink with a nonprogressive form of AD. Response to the virus of AD was significantly greater in PBL cultures from mink with nonprogressive AD than in those from normal mink or mink with progressive AD. After experimental infection with AD virus, mink PBL were responsive to viral antigen only transiently. These findings suggest that lymphocyte responsiveness as indicated by transformation induced by mitogens or viral antigen may be an important aspect of host response to infection with the parvovirus of AD.

Aleutian disease (AD) of mink has a complex pathogenesis which involves host immune response to a persistently infecting parvovirus (ADV). Disease is associated with a dramatic proliferation of plasma cells resulting in high levels of antiviral antibody and formation of virus-antibody immune complexes which induce fatal renal glomerular and vascular lesions (16). ADV is not neutralized by antibody, and vaccination with inactivated ADV as well as passively acquired antibody enhances disease signs, whereas immunosuppression with cyclophosphamide prevents development of AD (7, 15).

The outcome of infection with ADV is influenced by host genotype, since mink which are homozygous for the recessive Aleutian gene invariably die after experimental or natural infection, whereas animals which are not homozygous may survive challenge with some strains of ADV (11, 12). An analogous situation exists in ranned mink, in which infection with ADV has been found to follow progressive (P-AD) and nonprogressive (NP-AD) courses in individual animals. These two types of natural disease are differentiable on the basis of clinical, immunological, and pathological criteria (2). Mink with NP-AD, in contrast to those with P-AD, develop only moderate elevation of plasma gamma globulin and specific antibody to ADV, and their survival is apparently normal (2, 3). Microscopic lesions typical of AD are not found in mink with NP-AD (3). Although infection can be transmitted from mink with NP-AD and produce P-AD in contacts, transmission is less easy than from animals which have P-AD, and it is likely that virus titers are lower in mink with NP-AD than in those with P-AD (3).

Since survival of ADV-infected mink cannot be due to non-neutralizing virus-specific antibody (3, 5), the experiments described here were undertaken to investigate and compare cell-mediated immunity (CMI) in mink with P-AD and NP-AD. Defective T-lymphocyte function in mink with P-AD has been reported (14), and possible differences in activity of CMI may occur between normal mink and mink with P-AD or NP-AD.

MATERIALS AND METHODS

Animals. Normal Aleutian (aa), pastel (AA or AA), and standard dark (AA) mink were obtained from a ranch which is historically free of AD. Pastel mink naturally infected with ADV were obtained from commercial mink ranches. Mink were classified as normal (N), NP-AD, or P-AD on the basis of serum antibody to ADV detected by counterimmunoelectrophoresis (absent in N; ≤1:640 in NP-AD; ≥1:640 in P-AD) and of serum gamma globulin levels (≤20% of TP in N and NP-AD; ≥25% of TP in P-AD). A number of mink with NP-AD were held in isolation for a minimum of 5 years, during which time they remained healthy and positive for serum antibody to ADV. The blood of these minks was capable of inducing P-AD in N mink. Selected mink of this NP-AD group were killed and at necropsy did not have lesions of AD. Criteria for differentiation of N, NP-AD, and P-AD have been described (1, 2).

ADV. The ADV virus strain Guelph was used (10) as antigen and for infection of mink. The virus was obtained from pooled tissues of infected mink by fluo-
rocarbon treatment and centrifugation (9). A saline suspension of ADV containing 10⁶.⁰ mink 50% infectious doses per ml was used to infect mink by intraperitoneal injection. For use in lymphocyte transformation, a virus pool was titrated by counterimmunoelectrophoresis (8) against a standard pool of mink sera with antibody activity for ADV. One unit of ADV antigen activity was defined as the minimum concentration of ADV suspension which produced a clear precipitin reaction with a 1:40 dilution of standard positive serum.

Lymphocyte cultures. Mink were bled from the jugular vein into glass tubes containing ethylenediaminetetraacetic acid and lymphocytes were obtained after centrifugation of 1.5 volumes of blood on 1 volume of Ficoll-Paque (Pharmacia Ltd., Dorval, Quebec) by the method of Boyum (6). Recovered cells which were found to be 95% peripheral blood lymphocytes (PBL) were washed and suspended to a concentration of 10⁸/ml in Eagle minimal essential medium (GIBCO, Grand Island, N.Y.) containing penicillin (100 U/ml), streptomycin (50 μg/ml), and NaHCO₃ (0.25 ml of 7.5% solution per 100 ml of Eagle medium). Fetal calf serum was added to a final concentration of 15% for mitogen-induced transformation assays and 20% for ADV-induced transformation assays. One millilitre of cell suspension was incubated in polystyrene tubes (12 by 76 mm; Falcon Plastics, Oxnard, Calif.) for various periods at 37°C in a humid atmosphere of 5% CO₂.

Lymphocyte transformation. Mitogens were added to 1.0 ml of PBL cultures in 0.1-ml volumes at various concentrations and for incubation times of 96 h, to be optimal for inducing transformation of PBL from NP-AD mink. For stimulation by mitogens, cultures were maintained for 3 days; 7-day culture periods were used for lymphocyte stimulation with ADV. To detect lymphocyte transformation, 1 μCi of [methyl-¹H]thymidine (19 Ci/mmol; Amersham Corp., Oakville, Ontario) was added in 0.1 ml of Eagle medium to each culture 12 h before the lymphocytes were collected by cell harvester (Titertek; Flow Laboratories, Inc., Rockville, Md.). Isotope incorporation was measured by scintillation counting, and the results were expressed as counts per minute of the difference between arithmetic mean values from triplicate stimulated and unstimulated cell cultures. ADV was added as antigen to lymphocyte cultures at 0.1, 0.5, 1.0, and 0.01 antigen units/10⁸ lymphocytes. Phytoschlamagglutinin (PHA-P; Difco Laboratories, Detroit, Mich.) and concanavalin A (Con-A; Sigma Chemical Co., St. Louis, Mo.) were added at a ratio of 10 μg/10⁸ lymphocytes. Pokeweed mitogen (PWM; Sigma) was added to lymphocyte cultures in 0.1 ml of a 1:20 dilution of PWM solution.

Experimental design. PBL were obtained from 43 normal 1- to 2-year-old male and female mink; 12 were aa, 16 were AA, and 12 were aa or AA. Response of PBL to PHA was determined and compared between color phases, and mean response was compared with that of 52 pastel mink with NP-AD and 32 pastel mink with P-AD. Twenty of the normal mink were used to determine response of their PBL to Con-A and to compare this response with that of PBL from 23 3-year-old male and female pastel mink with NP-AD and 20 male and female 1- to 2-year-old mink with P-AD. Response to PWM was determined in PBL cultures of eight normal male and female 1-year-old mink, 15 3-year-old male and female mink with NP-AD, and 15 1- to 2-year-old mink with P-AD. A group of nine normal mink were infected by intraperitoneal injection of 10⁷ mink 50% infectious doses of ADV, and their PBL response to the optimal dose of ADV antigen was determined at 1, 2, 3, 5, and 7 weeks postinfection.

Statistical analysis. Differences between mean counts per minute for groups were evaluated for statistical significance by Student's t test.

RESULTS

Statistical comparison of mitogen-induced lymphocyte transformation between color phases (aa, AA, and AA) of normal mink failed to reveal significant differences. Likewise, results were not significantly influenced by the sex or age of the mink between the limits of 1 and 3 years. For these reasons, we considered mink of the same treatment groups and AD status to be a single group regardless of age and color phase. PBL responses to mitogens were similar, and mean counts per minute were not significantly different (P < 0.2–0.5) for normal and NP-AD mink, but mean counts per minute for both NP and AD groups were significantly greater (P < 0.01 to 0.001) than the means for mink with P-AD (Table 1).

Response to ADV antigen by PBL from mink with NP-AD consistently resulted in higher counts per minute than from unstimulated control lymphocyte cultures (Table 2). Values were low in comparison with those obtained from mitogen-stimulated PBL cultures (Table 1). In both N and P-AD mink, PBL cultured together with ADV were not stimulated and most frequently had lower incorporation of [methyl-¹H]thymidine than did unstimulated control cultures (Table 2). When mean values of counts per minute were compared between N and NP-AD mink, the values obtained from NP-AD mink were significantly greater (P < 0.05) for cultures

| Table 1. Transformation of mink PBL cultured with optimal doses of mitogens |
|---------------------------------|--------|--------|--------|
| AD status | PHA-P | Con-A | PWM |
| N         | 32,291 ± 4,485 | 53,115 ± 8,201 | 53,580 ± 6,644 |
| (43)      | (20)   | (8)    |
| NP-AD     | 31,389 ± 4,649 | 41,188 ± 5,109 | 50,303 ± 5,265 |
| (52)      | (23)   | (15)   |
| P-AD      | 16,189 ± 2,881 | 17,126 ± 5,590 | 14,191 ± 3,915 |
| (32)      | (20)   | (15)   |

* Values are mean ± standard error of the mean of the difference between PBL cultured with and without mitogen. Numbers in parentheses indicate numbers of mink.
stimulated with 1.0 U of ADV antigen but not with 0.01, 0.1, or 10.0 U (P < 0.1 to 0.50). Similarly, NP-AD mink PBL were significantly more responsive to ADV antigen than were PBL from P-AD mink at antigen doses of 0.1 and 1.0 U (P < 0.025 and 0.05). The difference was not significant (P < 0.2) at antigen doses of 0.01 and 10.0 U, respectively. PBL from mink with P-AD did not respond differently to ADV antigen than did PBL from N mink (P < 0.4–0.5).

After infection with ADV mink, PBL were significantly stimulated (P < 0.025) by culture with 0.1 antigen units of ADV in week 1 when mean counts per minute were compared with mean counts for week 0 (preinfection) (Table 3). The difference was not significant (P < 0.1 to 0.5) in weeks 2 to 7 after infection.

**DISCUSSION**

Results reported here confirm that lymphocytes of mink with P-AD have a depressed response to the T-lymphocyte mitogens PHA and Con-A (14). Response to the B-cell mitogen PWM is also subnormal. It has been reported previously that ADV-infected mink have a higher than normal proportion of surface immunoglobulin-bearing PBL (14) and that their antibody response to immunization with a variety of antigens is subnormal (13, 14). It is apparent, therefore, that in minks with P-AD the immune response and its regulation are distinctly abnormal, as anticipated from the observed proliferation of plasma cells, hypergammaglobulinemia, and excessive synthesis of ADV-specific antibody with resulting immune complex formation (16). Mink which are persistently infected with ADV but which fail to develop AD (NP-AD) have been shown here to possess PBL which are significantly more responsive to mitogens and to ADV antigen than are PBL of N mink or of mink with P-AD. In experimental infections with ADV, in vitro transformation of PBL in response to ADV antigens is transient, whereas in mink with NP-AD, PBL continue to transform when cultured with ADV several years after infection. CMI to viral antigen is often transient, diminishing as antibody synthesis reaches its peak. Antibody or antibody-virus complexes may inhibit CMI through a population of suppressor cells or by other mechanisms (4). It is not known whether the relatively lower antibody titers in NP-AD mink in comparison with P-AD mink may account for greater activity of CMI in this form of AD.

These results suggest that impaired T-cell function as indicated by poor response to PHA, Con-A, and ADV in P-AD mink might result in a progressive form of AD or, alternatively, impaired T-lymphocyte function may be caused by persistence of ADV and its complexes with antibody. Functional T-lymphocytes expressing CMI are known to be required for host resistance to disease produced by certain viruses, bacteria, fungi, protozoa, or metazoan parasites, and depression of CMI, often as a result of the infection itself, enhances disease production (4, 17).

Factors which determine the outcome of ADV infection of mink likely involve viral virulence and the ability of the host to control viral replication. In mink with NP-AD, the outcome of infection is favorable relative to that in mink with P-AD, but the relative resistance to disease in NP-AD can be overcome by superinfection with the Guelph strain of ADV (3). This strain does not produce NP-AD after laboratory infection but rather induces P-AD. Viral strain differences may occur, since the Pullman strain, which is regarded to be of relatively low virulence, induces NP-AD in a proportion of exper-
imentally infected pastel but not in sapphire mink (5). However, the Utah-1 strain, which is considered to be more virulent than the Pullman strain of ADV, has also been shown to induce NP-AD in approximately 25% of infected pastel mink (11). Mink identified on ranches to have NP-AD were shown to be a source of infection for normal mink which then developed P-AD (3). It is unlikely, therefore, that viral strain differences can account for the occurrence of P-AD or NP-AD, nor does the genotype determining color phase absolutely dictate the outcome of infection with ADV, although NP-AD does not apparently occur in Aleutian (aa) mink, whereas it is seen in a minority of ADV-infected non-Aleutian (aA, AA) mink.

ACKNOWLEDGMENTS
We thank Karen Saunders, Peter Wright, and Fausto De Pauli for their assistance. Financial support was received from The Canada Mink Breeders Association, Canada Department of Agriculture, and The Ontario Ministry of Agriculture and Food.

LITERATURE CITED