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Antiviral Antibodies Stimulate Production of Reactive Oxygen Species in Cultured Canine Brain Cells Infected with Canine Distemper Virus

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Canine distemper is characterized mainly by respiratory, enteric, and nervous symptoms. Infection of the central nervous system results in demyelination, to which inflammation has been shown to contribute significantly. It has been proposed that macrophages play a major role as effector cells in this process. We report that cultured dog brain cells contain a population of macrophages capable of producing reactive oxygen species as measured by luminol-dependent chemiluminescence. In cultures infected with canine distemper virus, a burst of reactive oxygen is triggered by antiviral antibody. This response depends on the presence of viral antigens on the surfaces of infected cells and is mediated by the interaction of antigen-bound antibody with Fc receptors on the macrophages. Since there is no evidence in vitro or in vivo that oligodendrocytes, the cells forming myelin, are infected, our observation supports the hypothesis that "innocent bystander killing" is important in demyelination caused by canine distemper virus. Reactive oxygen species released from macrophages may contribute to destruction of myelin.

Canine distemper is caused by a morbillivirus closely related to measles virus. Demyelination in the central nervous system is a well-known consequence of infection with canine distemper virus (CDV) (19, 23). Initially, demyelinating lesions occur in the absence of inflammation and are associated with viral replication in the glial cells of the white matter (43). Some animals develop chronic lesions in which inflammation becomes an important feature (38, 41, 46). Wisniewski et al. (46) noted that inflammatory lesions in the white matter are far more destructive than the initial noninflammatory demyelination. These authors proposed an "innocent bystander cell" mechanism as the cause of the chronic lesions and suggested that macrophages have an important effector role in this process. More recent studies have shown that inflammation in distemper coincides with the intrathecal production of antiviral antibodies (44) and with virus clearance from the brain (3). Taken together, the findings by Wisniewski et al. (46) and our own observations suggest that the antiviral immune response in which brain macrophages are important effector cells may contribute to brain tissue damage in chronic canine distemper.

Cell cultures derived from brain tissue contain numerous macrophages, also referred to as microglial cells. These cells have been shown to possess various functions that are also found for macrophages isolated from other tissues or derived from cultured monocytes. In particular, brain macrophages have Fc receptors on their surfaces and show strong phagocytic activity (8, 9, 15, 20, 32, 33, 47). They can be induced to express major histocompatibility complex type 2 antigens (39) and can be stimulated to release mediators such as interleukin-1 (14, 16) and tumor necrosis factor (13). Recently, rat (7, 15) and mouse (36) brain macrophages have been reported to produce reactive oxygen species (ROS) upon stimulation with a variety of agents, e.g., phorbol myristate acetate (PMA), opsonized zymosan, and antibody-coated bovine erythrocytes.

It was shown previously that certain viruses (e.g., Sendai virus) are capable of activating the production of ROS in phagocytic cells directly, i.e., by binding to the plasma membranes of phagocytes (25, 27, 28–30). In contrast, other viruses (e.g., herpesviruses) activate the generation of ROS indirectly through antiviral antibodies which link viral surface antigens with Fc receptors of the phagocytes (2, 45). The present study shows that antiviral antibodies trigger the generation of ROS in cell cultures infected with canine distemper virus. The production of ROS induced by antiviral antibodies in dog brain cell cultures may represent a host defense mechanism. In addition, ROS could damage noninfected cells such as oligodendrocytes.

MATERIALS AND METHODS

Dog brain cell cultures. Cultures of dog brain cells were prepared as described elsewhere (48). In brief, the cerebella of newborn dogs were aseptically removed immediately after euthanasia with an overdose of phenobarbital and were dissociated by chopping and repeated aspiration through 10-ml glass pipettes. The brain cell suspension was seeded in 10-cm plastic petri dishes (Falcon; Becton Dickinson Labware, Basel, Switzerland) containing 10 ml of Dulbecco modified Eagle medium (GIBCO, Basel, Switzerland) with 10% fetal calf serum, 250 U of penicillin per ml, and 1 μg of streptomycin per ml. Eight round glass cover slips 22 mm in diameter were attached to the bottoms of the petri dishes with silicon grease. The cultures were kept at 37°C in a CO_2 incubator (5% CO_2, 95% air). The culture medium was changed every 4 days. In some experiments, heparinized and anesthetized pups were perfused intracardially with Hanks balanced salt solution (HBSS) to wash the blood from the brain blood vessels, thus minimizing the presence of circulating blood monocytes in the brain cell cultures.

Infection with CDV. Confluent cultures of dog brain cells 7 to 14 days old were infected with virulent CDV strain A75/17 ($10^{2.5}$ 50% tissue culture infective doses per dish) (virus kindly donated by M. Appel, Cornell University, Ithaca, New York) as described elsewhere (50). Uninfected control cultures were derived from the same batch.

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Immunocytochemistry. Infection of the cell cultures was monitored with peroxidase-antiperoxidase (PAP) staining (37) for viral antigens by using a monoclonal antibody against the nucleoprotein of CDV (3). Astrocytes were detected with a polyclonal antiserum to glial fibrillary acidic protein (Dakopatts, Copenhagen, Denmark), and oligodendrocytes were stained with antiserum to myelin-associated glycoprotein (48). Macrophages were detected by examining their ability to form rosettes with immunoglobulin G (IgG)-coated erythrocytes (40). Bovine erythrocytes were opsonized with rabbit IgG to erythrocyte stroma (Nordic Immunological Laboratories, Tilburg, The Netherlands) as described for sheep erythrocytes (21). Erythrocytes coated with this antibody were added to unfixed cultures and incubated for 30 min at 37°C. The unbound erythrocytes were washed away before fixation and counterstaining. Uncoated erythrocytes served as the control.

Nitro Blue Tetrazolium assay. The cells were covered with a solution of Nitro Blue Tetrazolium (2 mg/ml in phosphate-buffered saline [PBS]) containing PMA (10^{-7} M) and KCN (10^{-3} M). After 15 min the solution was aspirated, and the cultures were fixed with 95% ethanol–5% acetic acid for 5 min at −20°C. Formation of the blue formazan granules was monitored by light microscopy.

In some experiments the erythrocyte-rosetting or Nitro Blue Tetrazolium assays were combined with immunocytochemical staining for glial fibrillary acidic protein or myelin-associated glycoprotein in uninfected cultures and for CDV antigens in infected cultures.

Subcultures derived from primary brain cell cultures. Macrophages were isolated from primary brain cell cultures as described elsewhere (12, 16). In brief, uninfected and infected primary brain cell cultures grown in 160-cm^2 tissue culture flasks (Costar, Mullingar, Ireland) were agitated on a rotary shaker (120 rpm for 30 min) 10 to 14 days after seeding and 3 to 4 weeks after infection, respectively. The supernatant was removed and centrifuged at 300 × *g* for 10 min at room temperature. The pellets were suspended and seeded in petri dishes containing cover slips. Nonadherent cells were removed after 30 min of sedimentation, and the remaining cells were identified with the erythrocyte-rosetting and Nitro Blue Tetrazolium assays. CDV-infected macrophages were stained simultaneously for virus antigens by means of the immunocytochemical methods described above.

Preparation of stimulating agents used in chemiluminescence assays. (i) Opsonized zymosan. Zymosan A (Sigma Chemical Co., St. Louis, Mo.) was mixed with 1 ml of fresh bovine serum and incubated at 37°C with continuous shaking for 30 min. After four washes in PBS by centrifugation at 1,000 × *g* and 4°C for 10 min, the pellet was resuspended in 1 ml of PBS and stored at −20°C.

(ii) PMA. PMA (Sigma) was dissolved in dimethyl sulfoxide at a concentration of 1 mM and further diluted in PBS as required.

(iii) CDV antibodies. Serum samples were obtained from three dogs after experimental infection with CDV strain A75/17 (42) and from one dog immunized with CDV strain Onderstepoort. Sera contained moderate to high titers (48 to 186) of neutralizing antibodies against CDV. Nonimmune sera served as the controls. IgG was purified by affinity chromatography with protein A-Sepharose (Pharmacia, Dübendorf, Switzerland) and by subsequent concentration by membrane ultrafiltration (PM 10; Amicon Corp., Wallisellen, Switzerland). The IgG fractions were pepsinized to obtain the $F(ab')_2$ fragments by using the method of Fey et al. (11). Protein concentrations of intact IgG and $F(ab')_2$ fractions were adjusted to 8 and 4 mg of PBS per ml, respectively. IgG and $F(ab')_2$ samples were stored at −20°C.

(iv) Immune complexes. Complexes of virus and antiviral antibody were prepared with CDV A75/17 propagated in primary dog brain cell cultures and purified by sucrose density gradient centrifugation (18). Virus (10^7 50% tissue culture infectious doses) suspended in 250 μl of TEN buffer (0.01 M Tris hydrochloride, 0.001 M EDTA, 0.1 M NaCl [pH 7.2]) was mixed with 750 μl of heat-inactivated immune serum (titer of antibodies to CDV, 186) (42). The samples were then incubated for 1 h at 37°C and kept at −20°C until used.

Rabbit antiserum to dog IgG $F(ab')_2$ was purchased from Nordic. For chemiluminescence (CL) assays, all serum samples described above and all antibodies [IgG, $F(ab')_2$, and immune complexes] were used at a final dilution of 1:100. The final concentration of opsonized zymosan was 100 μg/ml. PMA was used at end concentrations of 10^{-6} to 10^{-8} M. Sendai virus was added at 375 to 1,500 hemagglutinating units per vial, and CDV was added at 10^5 50% tissue culture infectious doses per vial. IgG-coated erythrocytes were incubated at a target (erythrocyte)-to-effector (brain cell) ratio of 20:1.

Hemadsorption assay for Sendai virus. Sendai virus, grown, purified, and titrated as described elsewhere (29), was added to the primary brain cell cultures and macrophage subcultures at a concentration of 375 hemagglutinating units per cover slip. After incubation for 10 min at 37°C, the cultures were rinsed five times with HBSS. To detect cell-associated virus, human erythrocytes suspended in PBS were added (10^7 erythrocytes per cover slip). After 15 min, nonadherent erythrocytes were washed away with PBS. Fixation of cultures was followed by staining for astrocytes and oligodendrocytes.

CL assay. Uninfected cell cultures were assayed for CL from day 0 through day 45 of culture. Light emission of infected cultures was measured when a near-confluent infection (as monitored by microscopic inspection) was established, usually between 3 and 4 weeks after infection. To prepare brain cell cultures or subcultured macrophages for CL assays, the cover slips were removed from the petri dishes and washed three times with PBS. The cover slips were then transferred to 25-mm glass scintillation vials (Tewis, Berne, Switzerland) containing 1.5 ml of HBSS with 5 mM glucose and 5 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma). In some experiments, 10 μg of microperoxidase (MP-11, no. M-6756; Sigma) per vial was added to amplify evoked CL, and in other experiments sodium azide (1 to 100 μM), an inhibitor of myeloperoxidase and eosinophil peroxidase (A. Jörg, J. M. Pasquier, and P. Portmann, Experientia **35**:936, 1979) was added to the assay buffer. Before the CL experiments were begun, the cells were dark adapted at 37°C for 10 to 20 min to reduce background CL. CL was measured at 37°C in a modified liquid scintillation spectrometer (Betamatic 1; Kontron, Zurich, Switzerland) with the settings for tritium in the out of coincidence mode. Light emission was counted repeatedly for 6-s periods twice before and 20 times after addition of the CL-inducing agents. Addition of the CL-inducing agents is indicated in the figures as zero time. CL data were recorded on line with a Hewlett-Packard Co. HP9816 desk computer.

RESULTS

Morphological observations (i) Uninfected brain cell cultures. Cultures became confluent 10 to 14 days after seeding

and contained many astrocytes and oligodendrocytes, as described previously (48). Throughout the culture period, 1 to 5% of the total cell population was identified as macrophages by a rosetting assay with IgG-coated erythrocytes. The macrophages were located mainly on top of the astrocyte layer (Fig. 1A to E). The majority of cells binding erythrocytes had the sombrerolike appearance described by Frei et al. (12); ramified-type macrophages (8, 13, 15) were only infrequently seen. IgG-coated erythrocytes did not bind to astrocytes and oligodendrocytes (Fig. 1B and C). Formazan granules were detected only in erythrocyte rosette-forming macrophages.

(ii) Infection of primary brain cell cultures with CDV. As described previously (50), the infection spread relatively slowly in the cell cultures and was noncytolytic. A nearly confluent infection was observed 3 to 4 weeks after infection. The majority of the macrophages forming rosettes with IgG-coated erythrocytes were infected 14 to 21 days after infection. Some of the infected macrophages and astrocytes underwent cell fusion, with formation of syncytia. Infected macrophages showed an increased tendency to form cell processes. At the advanced stages of infection, many macrophages were superimposed on and therefore were in close contact with other infected cells (Fig. 1E). The number of macrophages decreased during the infection.

(iii) Subcultures derived from primary mixed brain cell cultures. Subcultures of primary brain cell cultures consisted of more than 95% macrophages as enumerated in the rosetting assay. In uninfected subcultures, macrophages were round and had ruffled cell membranes. In contrast, CDV-infected macrophages were flattened, with little ruffling of the cell membranes, and often contained intracytoplasmic vacuoles (Fig. 1D). Many macrophages derived from infected primary cultures had become large and multinucleated in subculture as a result of CDV-induced cell fusion.

CL experiments. (i) Uninfected brain cell cultures. When exposed to opsonized zymosan, cultured dog brain cells produced CL similar to that described for cultured mouse brain cells (36). The burst of CL reached its maximum 5 to 10 min after the addition of zymosan and thereafter decreased slowly (data not shown). In contrast, addition of IgG-coated erythrocytes to the cultures resulted in a slower increase in light emission, with a peak at 30 to 40 min (Fig. 2). The kinetics of CL induced by PMA depended on the concentration of the stimulant: PMA at 10^{-6} M evoked CL peaking within 2 to 4 min (Fig. 3), whereas maximum light emission induced by 10^{-8} M PMA was reached between 30 and 40 min. Azide used at concentrations up to 100 μM failed to inhibit CL induced by PMA and zymosan. Elimination of blood from the brain by perfusion with HBSS before preparation of the cell cultures had no effect on the outcome of the CL experiments. The response to the CL-inducing agents increased with the age of the cultures, reaching a plateau between days 16 and 30 that was followed by a decline until day 45, when the experiment ended (data not shown). Sendai virus at concentrations of 375 to 1,500 hemagglutinating units per vial failed to evoke a burst of CL. To exclude the possibility that the failure to respond to Sendai virus was due to a lack of binding, we carried out a hemadsorption assay which detects cell-associated hemagglutinin. Microscopic examination of the cultures revealed that erythrocytes bound almost exclusively to macrophages (results not shown).

(ii) CDV-infected brain cell cultures. Infected cell cultures retained their ability to release ROS in response to all agents that were active in uninfected cultures, though with lower intensity (see Fig. 6 for the effect of zymosan). Inspection of the cultures showed that infection also led to a decrease in the number of macrophages. In infected but not in uninfected cultures, CL could be induced by adding CDV-specific immune serum (Fig. 4). Serum from unimmunized dogs failed to evoke CL in both uninfected and infected cultures (Fig. 4). The strongest CL response was obtained in the advanced stages of CDV infection of the culture, usually between 3 and 4 weeks after infection. Antiviral IgG induced a CL response similar to that of immune serum, while $F(ab')_2$ fragments prepared from antiviral IgG failed to evoke CL (Fig. 5). However, CL was stimulated when rabbit anti-dog IgG was added to such cultures after washing off unbound $F(ab')_2$. In this experiment, the Fc portion of rabbit antibody bound to dog anti-CDV $F(ab')_2$ was responsible for CL induction. This indicated that the lack of CL induction observed with dog anti-CDV $F(ab')_2$ alone was not due to the failure of $F(ab')_2$ fragments to bind to the viral antigen but reflected the role of the Fc portion in cell activation (Fig. 5). The rabbit antibody to dog IgG alone did not induce significant CL.

(iii) CL of subcultured macrophages. Subcultured uninfected and CDV-infected macrophages were seeded at a density of 10,000 to 50,000 cells per cover slip, thus avoiding contact between individual cells. These cultures produced CL with kinetics similar to those of primary brain cell cultures when triggered with opsonized zymosan (Fig. 6), PMA, and IgG-coated erythrocytes (data not shown). In addition, macrophages generated a burst of CL when stimulated with virus-immune complexes but not virus or immune serum alone (Fig. 7).

DISCUSSION

The results demonstrate that antiviral antibodies stimulate production of ROS in cultured dog brain cells infected with CDV. The mechanism of ROS production depends on viral antigen expressed on the surfaces of infected cells linked with Fc receptors on macrophagelike responder cells. Production of ROS in response to antiviral antibody was assessed by measuring luminol-dependent CL. This highly sensitive technique reflects the generation of various ROS (1). Others have shown that light emission closely reflects the formation of O_2^- in cultured rat and mouse brain cells (14, 36). Because cell cultures derived from the brains of newborn animals are composed of several cell types, it was important to define the cellular origin of ROS in our system. Several observations argue against the small possibility that we were measuring a signal emanating from leukocytes present in brain blood vessels and not removed at the time when the cultures were prepared. (i) Inspection by light microscopy failed to provide any evidence for the presence of blood leukocytes in the cultures when the experiments were performed. (ii) Perfusion with HBSS before the cultures were established failed to decrease CL in cultured dog brain cells, and the CL signal increased rather than decreased during the culture period. (iii) The CL signal was largely resistant to azide, an inhibitor of myeloperoxidase and eosinophil peroxidase (A. Jörg, J. M. Pasquier, and P. Portmann, Experientia **35**:936, 1979). This excluded neutrophilic leukocytes, monocytes, and eosinophils as a significant source of ROS in the cell cultures. (iv) Sendai virus, a potent activator of CL generation in polymorphonuclear leukocytes and monocytes, failed to evoke a response in cultured dog brain cells.

The approach chosen to more precisely identify ROS-producing cells in the cultures involved a combination of

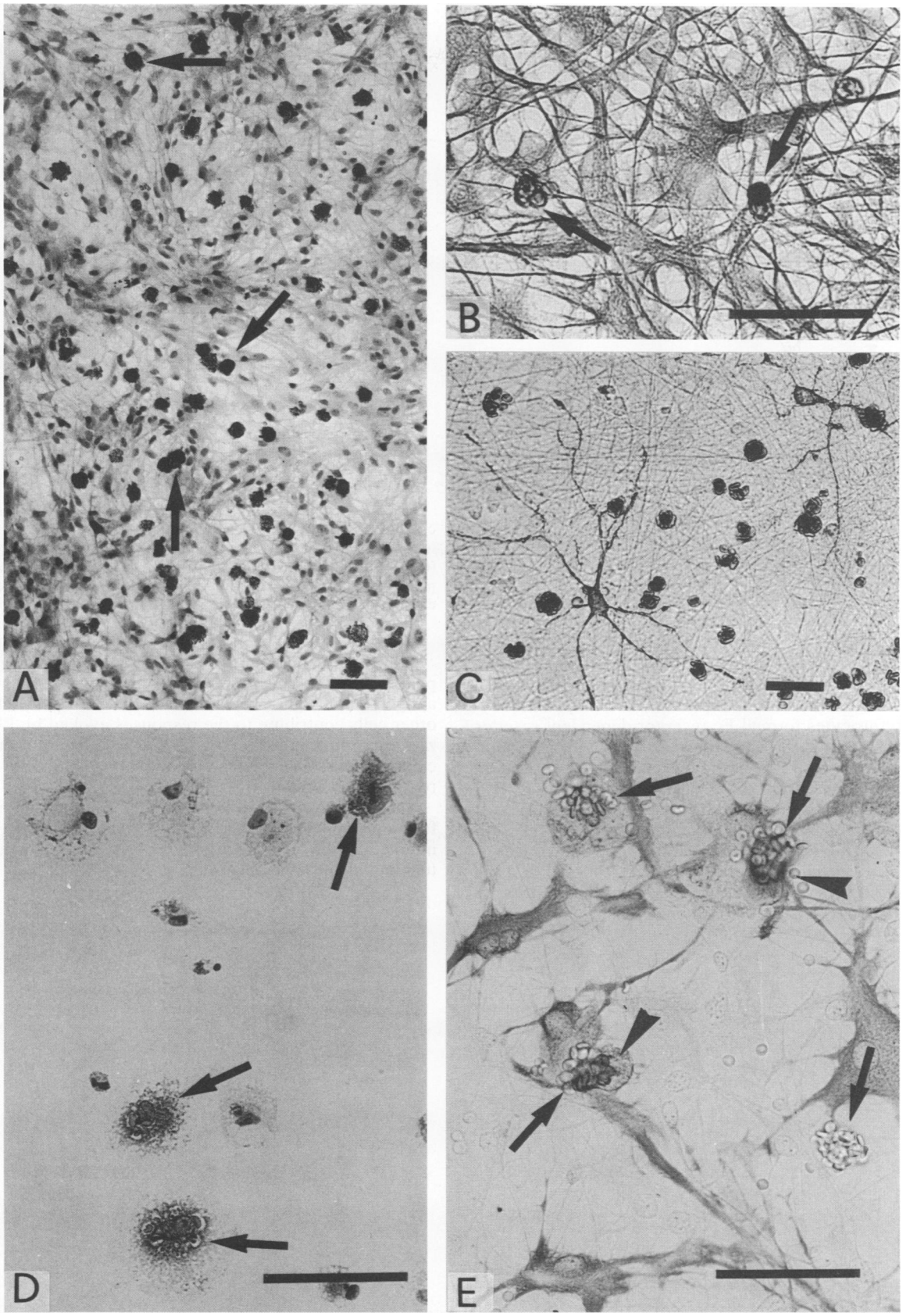

FIG. 1. Light microscopy of dog brain cell cultures. (A) Erythrocyte-rosetting assay in primary cell culture 2 weeks after seeding. A few of the macrophages binding IgG-coated erythrocytes are marked by arrows. (B) Erythrocyte-rosetting assay combined with immunocytochemical staining for astrocytes (rabbit anti-glial fibrillary acidic protein-PAP). IgG-coated erythrocytes do not bind to astrocytes. Two macrophages binding erythrocytes are marked by arrows. (C) Erythrocyte-rosetting assay combined with immunocytochemical staining for oligodendrocytes (human anti-myelin-associated glycoprotein-PAP). No binding of opsonized erythrocytes to oligodendrocytes is visible. Note the numerous macrophages binding erythrocytes. (D) Cultured macrophages isolated from primary brain cell cultures infected with CDV. Arrows indicate virus-infected macrophages (mouse anti-nucleoprotein of CDV-PAP). (E) Erythrocyte-rosetting assay combined with immunocytochemical staining for CDV antigen (mouse anti-nucleoprotein of CDV-PAP). Four macrophages (arrows) are in close contact with other virus-infected brain cells. Two of these macrophages (arrowheads) are also positive for CDV antigen. Bars, 100 μm.

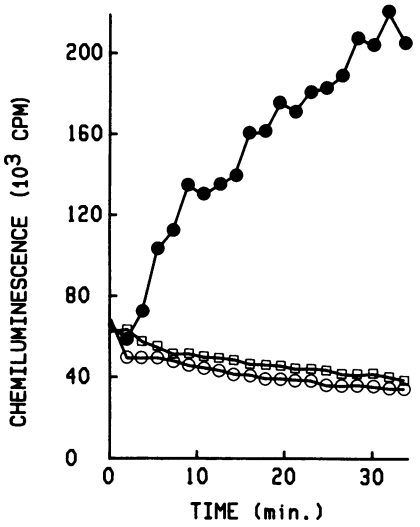

FIG. 2. Luminol-dependent CL triggered by IgG-coated bovine erythrocytes in dog brain cells cultured for 17 days. Results are shown for IgG-coated erythrocytes (●), uncoated control erythrocytes (○), and antibody alone (□). Each datum point represents the mean for two samples run in parallel. Erythrocytes were added at zero time.

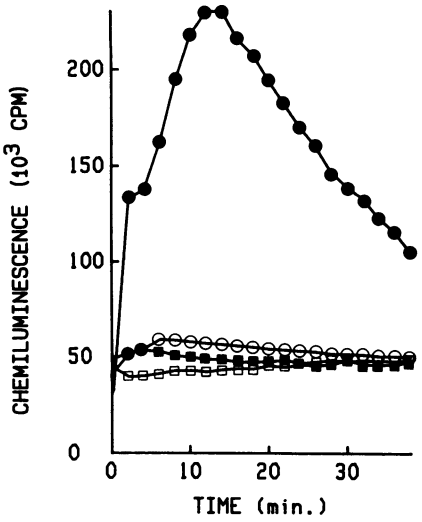

FIG. 4. CL triggered by dog sera in CDV-infected and uninfected cultured dog brain cells. Shown are the effects of dog anti-CDV immune serum on CL in infected cultures 20 days after infection (●), of the same serum in uninfected cultures (■), and of nonimmune serum in infected (○) and uninfected (□) cultures. Each datum point represents the mean for two samples run in parallel.

cytochemical and rosetting assays. The macrophages were clearly positive in the Nitro Blue Tetrazolium assay when stimulated with PMA. Most important, however, antibody-coated erythrocytes formed Fc receptor-dependent rosettes with macrophages only. Since these erythrocytes also stimulated a burst of ROS in brain cell cultures, the rosetting assay clearly identified macrophages as the source of ROS production triggered by antibody. It is of particular interest that Sendai virus bound predominantly to these cells but failed to evoke CL. Since the CL of blood monocytes is largely sensitive to inhibition by azide, this suggests that the brain macrophages responsible for the generation of ROS are not directly derived from blood monocytes.

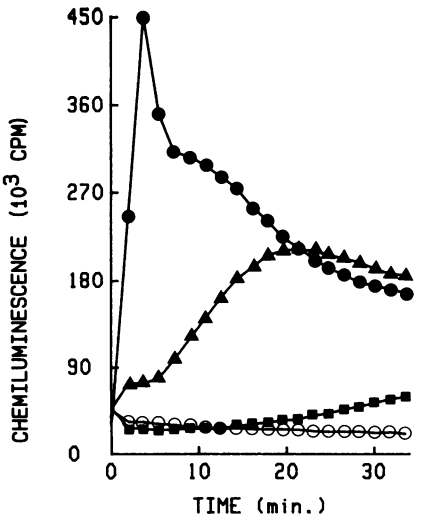

FIG. 3. Effect of PMA on luminol-dependent CL in dog brain cells cultured for 14 days. PMA was added to the cells to final concentrations of 10^{-6} M (●), 10^{-7} M (▲), and 10^{-8} M (■). Light emission by cells mock-stimulated by PBS is also shown (○). Each datum point represents the mean for two samples run in parallel.

While our experiments clearly showed that brain macrophages are the source of ROS produced in response to antigen-bound IgG, we cannot exclude the possibility that other cells contribute to the responses evoked by PMA and zymosan. Resolution of this issue is difficult because no clear-cut assays showing binding and activation concurrently are available.

The mechanism by which antiviral antibody induced the formation of ROS in macrophages was dependent on the interaction between the antibody Fc portion and Fc receptors on the one hand and on the presence of antigens of CDV

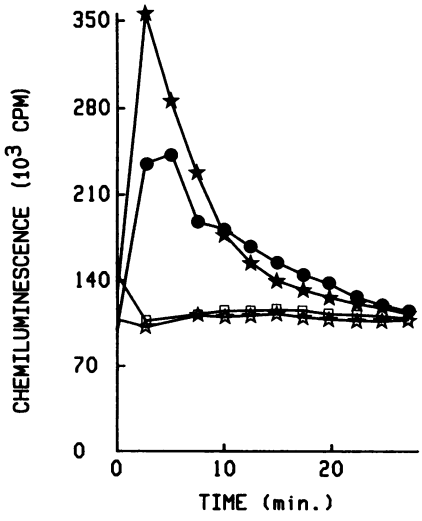

FIG. 5. Role of the Fc fragment in induction of CL in CDV-infected cultured dog brain cells 20 days after infection. Symbols: ●, effect of purified IgG from CDV immune dog serum; □, $F(ab')_2$ fragment prepared from this antibody; ★, rabbit antibody to dog $F(ab')_2$ fragment added after washing off unbound $F(ab')_2$; ☆, same antibody in cells not pretreated with $F(ab')_2$. Each datum point represents the mean for two cultures run in parallel.

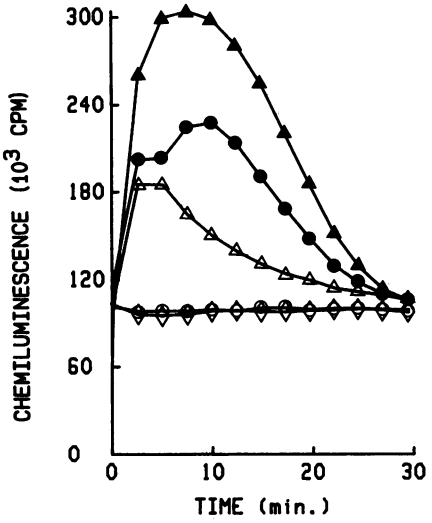

FIG. 6. Effects of dog anti-CDV immune serum and zymosan on CL of infected cultured dog brain cells and macrophages isolated from these cultures. Cell cultures prepared from the brains of newborn dogs were infected with CDV at day 14 of culture. Macrophages were isolated from these cultures 22 days after infection. Shown are the effects of immune serum added to primary cell culture (●), immune serum in isolated macrophages (○), zymosan in primary culture (▲), zymosan in isolated macrophages (△), and nonimmune serum in uninfected cultured dog brain cells. Each datum point represents the mean for two samples run in parallel.

virus on the other (Fig. 4 and 5). Virus-immune complexes are capable of activating the generation of ROS in macrophages (Fig. 7). However, in the experiments represented in Fig. 4 and 5, viral antigens present in the cell culture supernatant were removed before the addition of antibody, arguing against soluble immune complexes as the trigger of ROS formation. The most likely possibility is that production of ROS was activated by antibodies linking viral antigens on the surfaces of virus-infected cells with Fc receptors on macrophages. In dog brain cell cultures, astrocytes and macrophages but not oligodendrocytes have been infectible with CDV (50). The observation that infected macrophages failed to respond to stimulation with antiviral antibody after isolation from the primary cell cultures and seeding at a density minimizing close contact between neighboring cells argues against "self-triggering" (i.e., bridging viral antigen and Fc receptor on the same cell) as an important mechanism in ROS formation. From the close topological association between brain macrophages and astrocytes, we assume that ROS formation is activated in a manner similar to that of antibody-dependent, cell-mediated cytotoxicity, with astrocytes serving as the target cells.

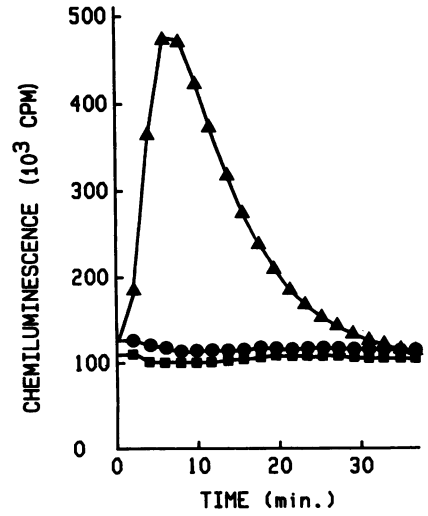

FIG. 7. Effect of CDV-immune complexes on CL in macrophages isolated from dog brain cell cultures. Macrophages were isolated from a 24-day brain cell culture. Symbols: ▲, macrophages stimulated with CDV-immune complexes; ■, CDV virus alone; ●, dog anti-CDV immune serum alone. Each datum point represents the mean for two samples run in parallel.

Brain cell cultures have been instrumental in studying the interaction between neurotropic viruses and cells in central nervous tissue (10, 17, 24). The experiments reported here demonstrate that these cell cultures allow study of the interaction between endogenous brain macrophages and virus-infected target cells. The demonstration that endogenous brain macrophages can be activated by antiviral antibody to produce ROS in response to viral antigens is of interest for several reasons. From an analytical viewpoint, the well-documented high sensitivity of antibody-induced CL techniques (for a review, see reference 29) makes it possible to study the expression of viral antigens that are direct targets for antibody-directed macrophages in brain tissue. This approach will be particularly interesting with viruses that restrict the expression of genes coding for surface antigens.

The biological consequences of ROS production by brain macrophages in response to viral antigens are not known. Toxic effects of some ROS are well documented (4). In antibody-dependent cytotoxicity mediated by mouse macrophages, ROS are known to contribute to target cell killing (26), and toxic effects of ROS have also been demonstrated in innocent bystander cells exposed to human macrophages (34). Since inflammatory demyelination in chronic distemper coincides with intrathecal production of anti-CDV antibodies, our observations in vitro support the hypothesis that tissue damage in chronic distemper results from an innocent bystander mechanism rather than directly from the antiviral immune response.

The mechanism of demyelination observed in these dogs is of particular relevance to this hypothesis. Oligodendrocytes, the cells forming myelin, degenerate without evidence of viral replication (49, 50). We have observed that oligodendrocytes are exquisitely sensitive to O_2^- generated by xanthine/xanthine oxidase (C. Griot, R. Stocker, A. Richard, E. Peterhans, and M. Vandevelde, submitted for publication). Moreover, it seems likely that the binding to Fc receptors of antiviral antibodies linked to viral surface antigens also activates other regulatory and effector functions in brain macrophages, such as the production of monokines (e.g., tumor necrosis factor and interleukin-1). It may be that an array of effector functions damage uninfected tissue and thus cause "autotoxicity" (31). In fact, ROS have been shown to degrade phospholipids in brain cortical slices (5), to damage myelin membrane proteins (22), and to play a role in the aging of myelin (6). Moreover, tumor necrosis factor was recently shown to mediate myelin and oligodendrocyte damage in vitro (35). Additional studies of the function of brain macrophages in cultured dog brain cells infected with CDV might help to define more precisely the inflammatory response that greatly aggravates the symptoms caused by neurotropic virus strains.

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LITERATURE CITED

1. **Allen, R. C.** 1986. Phagocytic leukocyte oxygenation activities and chemiluminescence: a kinetic approach to analysis. Methods Enzymol. **133**:449–493.
2. **Bingham, E. L., T. W. Fenger, A. Sugar, and J. W. Smith.** 1985. Dependence on antibody for induction of chemiluminescence in polymorphonuclear leukocytes by herpes simplex virus. Invest. Ophthalmol. Visual Sci. **26**:1236–1243.
3. **Bollo, E., A. Zurbriggen, M. Vandevelde, and R. Fankhauser.** 1986. Canine distemper virus clearance in chronic inflammatory demyelination. Acta Neuropathol. **72**:69–73.
4. **Cerutti, P. A., I. Fridovich, and J. M. McCord (ed.).** 1988. Oxy-radicals in molecular biology and pathology. UCLA Symp. Mol. Cell. Biol. **82**:543–562.
5. **Chan, P. H., M. Yurko, and R. A. Fishman.** 1982. Phospholipid degradation and cellular edema induced by free radicals in brain cortical slices. J. Neurochem. **38**:525–531.
6. **Chia, L. S., J. E. Thompson, and M. A. Moscarello.** 1983. Disorder in human myelin induced by superoxide radical: an in vitro investigation. Biochem. Biophys. Res. Commun. **117**: 141–146.
7. **Colton, C. A., and D. L. Gilbert.** 1987. Production of superoxide anions by a CNS macrophage, the microglia. FEBS Lett. **223**:284–288.
8. **Ditrich, H.** 1986. Brain macrophages in cerebellar cell cultures. Tissue Cell **18**:645–658.
9. **DuBois, J. H., G. D. Hammond-Tooke, and M. L. Cuzner.** 1985. The phagocytes of neonate rat primary mixed glial cell cultures. Int. J. Dev. Neurosci. **3**:531–539.
10. **Dubois-Dalcq, M., E. W. Doller, M. V. Haspel, and K. V. Holmes.** 1982. Cell tropism and expression of mouse hepatitis viruses (MHV) in mouse spinal cord cultures. Virology **119**: 317–331.
11. **Fey, H., H. Pfister, J. Messerli, N. Sturzenegger, and F. Grolimund.** 1976. Methods of isolation, purification and quantitation of bovine immunoglobulins. Zentralbl. Veterinaermed. Reihe B **23**:269–300.
12. **Frei, K., S. Bodmer, C. Schwerdel, and A. Fontana.** 1986. Astrocyte-derived interleukin 3 as a growth factor for microglial cells and peritoneal macrophages. J. Immunol. **137**:3521–3527.
13. **Frei, K., C. Siepl, P. Groscurth, S. Bodmer, C. Schwerdel, and A. Fontana.** 1987. Antigen presentation and tumor cytotoxicity by interferon-γ-treated microglial cells. Eur. J. Immunol. **17**: 1271–1278.
14. **Giulian, D.** 1987. Ameboid microglia as effectors of inflammation in the central nervous system. J. Neurosci. Res. **18**: 155–171.
15. **Giulian, D., and T. J. Baker.** 1980. Characterisation of ameboid microglia isolated from developing mammalian brain. J. Neurosci. **6**:2163–2178.
16. **Giulian, D., T. J. Baker, L. N. Shih, and L. B. Lachman.** 1986. Interleukin 1 of the central nervous system is produced by ameboid microglia. J. Exp. Med. **164**:594–604.
17. **Graves, M. C., L. Bologa, L. Siegel, and H. Loude.** 1986. Theiler's virus in brain cell cultures: lysis of neurons and oligodendrocytes and persistence in astrocytes and macrophages. J. Neurosci. Res. **15**:491–501.
18. **Hall, W. W., R. A. Lamb, and P. W. Choppin.** 1980. The polypeptides of canine distemper virus: synthesis in infected cells and relatedness to the polypeptides of other morbilliviruses. Virology **100**:433–449.
19. **Innes, J. R. M., and L. Z. Saunders.** 1962. Comparative neuropathology, p. 373–384. Academic Press, Inc., New York.
20. **Jordan, F. L., and W. E. Thomas.** 1987. Identification of microglia in primary cultures of mixed cerebral cortical cells. Brain Res. Bull. **19**:153–159.
21. **Jungi, T. W., and E. Peterhans.** 1988. Change in the chemiluminescence reactivity pattern during in vitro differentiation of human monocytes to macrophages. Blut **56**:213–220.
22. **Konat, G. W., and R. C. Wiggins.** 1985. Effect of reactive oxygen species on myelin membrane proteins. J. Neurochem. **45**:1113–1118.
23. **Krakowka, S., M. K. Axthelm, and G. C. Johnson.** 1985. Canine distemper virus, p. 137–164. *In* R. G. Olsen, S. Krakowka, and J. B. Blakeslee, Jr. (ed.), Comparative pathobiology of viral disease, vol. 2. CRC Press, Inc., Boca Raton, Fla.
24. **Massa, P. T., H. Wege, and V. ter Meulen.** 1988. Growth pattern of various JHM coronavirus isolates in primary rat glial cell cultures correlates with differing neurotropism in vivo. Virus Res. **9**:133–144.
25. **Mills, E. L., Y. Debets-Ossenkopp, H. A. Verbrugh, and J. Verhoef.** 1981. Initiation of the respiratory burst of human neutrophils by influenza virus. Infect. Immun. **32**:1200–1205.
26. **Nathan, C., and Z. Cohn.** 1980. Role of oxygen-dependent mechanisms in antibody-induced lysis of tumor cells by activated macrophages. J. Exp. Med. **152**:198–208.
27. **Peterhans, E.** 1979. Sendai virus stimulates chemiluminescence in mouse spleen cells. Biochem. Biophys. Res. Commun. **91**: 383–392.
28. **Peterhans, E.** 1980. Chemiluminescence: an early event in the interaction of Sendai and influenza viruses with mouse spleen cells. I. The role of the envelope glycoproteins in the stimulation of chemiluminescence. Virology **105**:445–455.
29. **Peterhans, E.** 1987. Virus- and antibody-induced chemiluminescence, p. 59–91. *In* K. Van Dyke and V. Castranova (ed.), Cellular chemiluminescence, vol. 2. CRC Press, Inc., Boca Raton, Fla.
30. **Peterhans, E., T. Baechi, and J. Yewdell.** 1983. Evidence for different receptor sites in mouse spleen cells for the Sendai virus hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. Virology **128**:366–376.
31. **Peterhans, E., T. W. Jungi, and R. Stocker.** 1988. Autotoxicity and reactive oxygen in viral disease. UCLA Symp. Mol. Cell. Biol. **82**:543–562.
32. **Raedler, E., and A. Raedler.** 1984. Local proliferation of brain macrophages in central nervous system tissue cultures. J. Neuropathol. Exp. Neurol. **43**:531–540.
33. **Raff, M. C., K. L. Fields, S.-J. Hakomori, R. Mirsky, R. M. Pruss, and J. Winter.** 1979. Cell type specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. Brain Res. **174**:283–308.
34. **Sagone, A. L., and J. J. Rinehart.** 1984. Human monocyte to macrophage differentiation in vitro: characterization and mechanisms of the increased antibody-dependent cytotoxicity associated with differentiation. J. Leukocyte Biol. **35**:217–228.
35. **Selmaj, K. W., and C. S. Raine.** 1988. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. Ann. Neurol. **23(4)**:339–346.
36. **Sonderer, B., P. Wild, R. Wyler, A. Fontana, E. Peterhans, and M. Schwyzer.** 1987. Murine glial cells in culture can be stimulated to generate reactive oxygen. J. Leukocyte Biol. **42**: 463–473.
37. **Sternberger, L. A.** 1979. Immunocytochemistry, p. 122–129. John Wiley & Sons, Inc., New York.
38. **Summers, B. A., H. A. Graisen, and M. J. G. Appel.** 1979. Early events in canine distemper demyelinating encephalomyelitis. Acta Neuropathol. **46**:1–10.
39. **Suzumura, A., S. G. E. Mezitis, N. K. Gonatas, and D. H. Silberberg.** 1987. MHC antigen expression on bulk isolated macrophage-microglia from newborn mouse brain: induction of Ia antigen expression by γ-interferon. J. Neuroimmunol. **15**: 263–278.
40. **Taffet, S. M., and S. W. Russel.** 1981. Identification of mononuclear phagocytes by ingestion of particulate materials such as erythrocytes, carbon, zymosan, or latex, p. 283–293. *In* D. O. Adams, P. J. Edelson, and H. Koren (ed.), Methods for studying mononuclear phagocytes. Academic Press, Inc., New York.
41. **Vandevelde, M., R. Fankhauser, F. Kristensen, and B. Kris-**

tensen. 1981. Immunological and pathological findings in demyelinating encephalitis associated with canine distemper virus infection. Acta Neuropathol. **54:**31–41.

42. **Vandevelde, M., R. J. Higgins, B. Kristensen, F. Kristensen, A. J. Steck, and U. Kihm.** 1982. Demyelination in experimental canine distemper virus infection: immunological, pathologic, and immunohistological studies. Acta Neuropathol. **56:**285–293.
43. **Vandevelde, M., A. Zurbriggen, R. J. Higgins, and D. Palmer.** 1985. Spread and distribution of viral antigen in nervous canine distemper. Acta Neuropathol. **67:**211–218.
44. **Vandevelde, M., A. Zurbriggen, A. Steck, and P. Bichsel.** 1986. Studies on the intrathecal humoral immune response in canine distemper encephalitis. J. Neuroimmunol. **11:**41–51.
45. **Weber, L., and E. Peterhans.** 1983. Stimulation of chemiluminescence in bovine polymorphonuclear lecocytes by virus-antibody complexes and by antibody-coated infected cells. Immunobiology **164:**333–342.
46. **Wisniewski, H., C. S. Raine, and W. J. Kay.** 1972. Observations on viral demyelinating encephalomyelitis. Canine distemper. Lab. Invest. **26:**589–599.
47. **Zucker-Franklin, D., A. Warfel, G. Grusky, B. Frangione, and D. Teitel.** 1987. Novel monocyte-like properties of microglial/astroglial cells. Lab. Invest. **57:**176–185.
48. **Zurbriggen, A., and M. Vandevelde.** 1984. Morphological and immunocytochemical characterisation of mixed glial cell cultures derived from neonatal canine brain. Res. Vet. Sci. **36:** 270–275.
49. **Zurbriggen, A., M. Vandevelde, and E. Bollo.** 1987. Demyelinating, non-demyelinating and attenuated canine distemper virus strains induce oligodendroglial cytolysis in vitro. J. Neurol. Sci. **79:**33–41.
50. **Zurbriggen, A., M. Vandevelde, and M. Dumas.** 1986. Secondary degeneration of oligodendrocytes in canine distemper virus infection in vitro. Lab. Invest. **54:**424–430.