Inhibitory Effects of Macrophages Against Marek's Disease Virus Plaque Formation in Chicken Kidney Cell Cultures [1, 2]

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ABSTRACT—Inhibition of plaque formation by Marek's disease virus (MDV) in chicken kidney cell cultures was investigated with the use of peritoneal exudate cells (PEC) from chickens. PEC from MDV-infected White Leghorn chickens inhibited the formation of MDV plaques, whereas the inhibitory effect of PEC from chickens vaccinated with herpesvirus of turkey (HVT) or PEC from normal chickens was very weak. However, PEC from either normal chickens or HVT-vaccinated chickens inhibited the MDV plaque formation in the presence of serum from MDV-infected chickens but not from normal or HVT-vaccinated chickens. The capacity of PEC to inhibit plaque formation was significantly reduced when PEC was treated with carrageenan but not with antithymus or antibursa cell serum. These results indicate that macrophages may have a role in protection against Marek's disease by reducing the number of MDV-infected cells and thereby decreasing the spread of the virus in vivo.—JNCI 63: 1267-1271, 1979.

MD is a lymphoproliferative disease of chickens caused by a herpesvirus (*1*). The host defense mechanisms against MDV infection and against tumor formation have been investigated (*2-5*), and several hypotheses have been proposed to explain protective mechanisms against MD by HVT vaccine (*6-9*). A two-step hypothesis of immunity against MD was postulated by Payne et al. (*7, 10*): The first step lowers the amount of virus in the bird, and the second step destroys neoplastically transformed cells, which leads to elimination of the tumor.

Studies of antiviral or antitumor immune mechanisms have been performed to clarify the role and type of effector cells against MDV-infected cells or MD-derived cell lines. PBL or spleen cells from MDV-infected or immunized birds are cytotoxic against MD-derived cell lines (*11-16*) and MDV-infected CKC (*17*). PBL also inhibit MDV plaque formation in CKC (*18*). Lymphocytes from HVT-vaccinated chickens also are cytotoxic against MD-derived cell lines (*19, 20*) and against MDV-infected CKC in the presence of MDV or HVT antiserum (*17*). However, the defense mechanisms against MD are complex, inasmuch as thymus-derived lymphocytes serve a dual role as target cells for MDV transformation and as cytotoxic effector cells against MD in host immune surveillance (*18, 21*). Furthermore, the exact role of other types of effector cells in MD immunity has not been fully clarified. In the present study, we describe the role of a macrophage in antiviral immunity and show that PEC from MDV-infected chickens inhibit MDV plaque formation.

MATERIALS AND METHODS

Chickens and virus inoculation.—Specific-pathogen-free White Leghorn chickens (line PDL-1) were used (*17*). They lacked maternal antibody to both MDV and HVT. The source of the JM strain of MDV and the FC 126 strain of HVT and the method of propagation of the virus in CKC cultures have been described (*22, 23*). A group of day-old chicks was inoculated ip with 0.1 ml heparinized peripheral blood from chickens infected with MDV (titer: 40 PFU/0.1 ml). Another group of chicks of the same age was vaccinated ip with 2,600 PFU/0.1 ml HVT-infected CKC. A third group of noninoculated chicks served as controls. Each group was kept in a separate room. Maximum precautions were taken to prevent cross-contamination among the 3 groups of chickens. The infection status was monitored in each group by direct kidney cultures, virus isolation from peripheral blood, and the AGP test (*17*).

PEC.—PEC were used as the effector cells for the plaque inhibition tests. Chickens that had been inoculated with MDV and showed severe MDV symptoms were excluded as a source of PEC. Chickens were inoculated ip with 40 ml 4% soluble starch. The chickens were killed 39-44 hours after inoculation, and PEC were collected by washing of the abdominal cavity with 100 ml PBS. The PEC were concentrated by centrifugation and resuspended in Eagle's basal medium (Nissui Seiyaku Co., Tokyo, Japan; pH 7.2) containing 1% FCS, 200 U penicillin/ml, 200 μg streptomycin/ml, and 2.5 μg Fungizone/ml. While we worked with PEC, all media and silicon-coated glassware were kept cold to minimize the chance of macrophage attachment. Viability of cells was 87.4±9.3% (mean±SD) as determined by the trypan blue dye exclusion test. PEC were composed of 71±11% macrophages, 16±9% granulocytes, and 14±6% lymphocytes as determined by Giemsa staining.

MDV plaque inhibition test.—The monolayers of CKC cultures on 35-mm plastic plates (#3001; Falcon

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ABBREVIATIONS USED: ADCC=antibody-dependent cellular cytotoxicity; AGP=agar gel precipitin; CI=cytotoxicity index; CKC=chicken kidney cell(s); FCS=fetal calf serum; HVT=herpesvirus of turkey; MD=Marek's disease; MDV=MD virus; PBL=peripheral blood lymphocytes; PBS=phosphate-buffered saline; PEC=peritoneal exudate cells; PFU=plaque-forming units.

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Plastics, Oxnard, Calif.) were inoculated with 180-390 PFU of the stock virus of cell-associated MDV. After 22-25 hours, cultures were washed once and varying numbers of PEC in 1 ml medium were added to the cultures. The cultures were incubated at 37° C in a 5% CO_2 humidified atmosphere. Medium changes were done every 2 days unless otherwise stated. In some experiments, heat-inactivated serum from MDV-infected, HVT-vaccinated, or normal chickens from the same lot was added to the cultures. MDV plaques were counted 4 days after addition of PEC. To determine the average plaque number for each sample, three or four replicate plates were used. Results were indicated by the percent of MDV plaques in test samples compared to the number of MDV plaques in cultures without PEC.

Treatment of PEC with carrageenan.—To determine effector cell type on MDV plaque inhibition, PEC treated with carrageenan (antimacrophage substance) (*24*) were added to the MDV-infected CKC cultures. PEC (8×10^6) from MDV-infected chickens were suspended in 1 ml culture medium containing 0.8-500 μg carrageenan/ml and incubated for 4 hours at 37° C. The treated cells were washed with culture medium three times, and 2×10^6 cells were added to the MDV-infected CKC cultures.

Treatment of PEC with antithymus or antibursa cell serum.—PEC (1×10^7 cells/0.1 ml) from MDV-infected chickens were incubated with 0.1 ml antithymus or antibursa cell serum (1:2) and 0.1 ml guinea pig complement (1:4) for 60 minutes at 37° C. The treated cells were washed three times with culture medium, and then 2×10^6 cells were added to the MDV-infected CKC.

Antithymus and antibursa cell serum.—For the preparation of antithymus and antibursa cell serum, thymus or bursa tissues collected from 10-day-old commercial chicks were processed by a Dounce-type homogenizer, and cell suspensions were washed three times with PBS. Rabbits were given injections iv of single-cell suspensions containing 2×10^8 cells twice at 2-week intervals. Sera were collected 2 weeks after the second injection and inactivated at 56° C for 30 minutes. Sera were absorbed three times with an equal volume of pooled chicken red blood cells and cross-absorbed three times with an equal volume of thymus or bursa cells.

Specificity of the sera was examined by the trypan blue dye exclusion test as described by Sugimoto et al. (*25*). Medium used for the test was RPMI-1640 (Nissui Seiyaku Co.; pH 7.4) containing 10% FCS. We mixed 25 μl thymus or bursa cell suspension (1×10^7 cells/ml), 25 μl twofold dilutions of antiserum, and 25 μl guinea pig complement (1:4) in the well of a microplate (#3040; Falcon Plastics) and incubated them at 37° C for 45 minutes. After incubation, 50 μl 0.16% trypan blue solution was added to each well, and we established the viability of cells immediately by counting a minimum of 150 cells. As controls, the complement and the antiserum were replaced by medium. CI was calculated by the following formula: CI=[(percent viable cells in cell suspension alone − percent viable cells in test sample)/percent viable cells in cell suspension alone]×100.

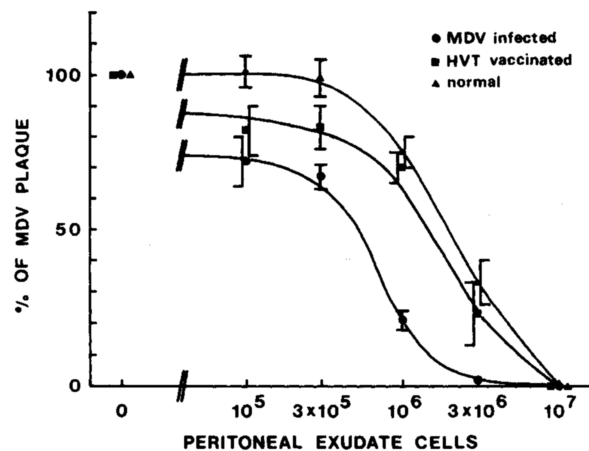
Sera.—Sera used in the experiments were collected from line PDL-1 chickens inoculated with MDV or vaccinated with HVT at 1 day of age or from normal chickens. Sera were stored at −35° C until use. All sera were inactivated at 56° C for 30 minutes before use and were examined for the presence of anti-MDV or anti-HVT antibody by the AGP test (*17*).

Statistical analysis.—Data were analyzed statistically with the use of Student's *t*-test.

RESULTS

Inhibition of MDV Plaque Formation by PEC

Text-figure 1 shows the inhibition of MDV plaque formation by PEC from MDV-infected, HVT-vaccinated, and normal chickens. Tests were repeated five times, and 2 chickens from each of the 3 groups of the same age (60, 74, 85, 95, and 102 days of age) were used in each experiment. In these tests, PEC contaminated by chicken red blood cells were excluded from the tests. The number of PEC samples tested was seven MDV infected, seven HVT vaccinated, and eight normal. Results indicated that PEC from MDV-infected birds markedly inhibited MDV plaque formation at cell concentrations of 1×10^5 to 3×10^6 per culture. This inhibitory effect was statistically significant when compared to that of PEC from normal chickens ($P<0.001$). An inhibitory effect was also observed with PEC from HVT-vaccinated or normal chickens when the number of PEC was increased. PEC from chickens vaccinated with HVT, however, did not show any statistically significant MDV plaque inhibition when compared to PEC from normal chickens.



TEXT-FIGURE 1.—Inhibition of MDV plaque formation by PEC. *Each symbol* represents PEC from MDV-infected chickens (No.=7), HVT-vaccinated chickens (No.=7), or normal chickens (No.=8). *Each point and vertical line* represent the mean value and SE from five separate tests.

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Effect of Treatment of PEC With Carrageenan

The effect of carrageenan treatment of PEC from an MDV-infected chicken (74 days old) is shown in text-figure 2. In this experiment, 1 ml medium was added every 2 days instead of the medium being changed. The MDV plaque inhibition by PEC was reduced by treatment with 100 μg carrageenan and thoroughly eliminated with 500 μg.

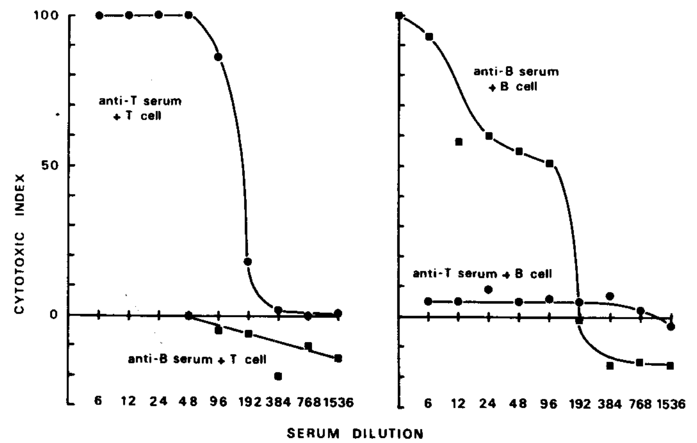
Effect of Treatment of PEC With Antithymus or Antibursa Cell Serum

The specificities of antithymus and antibursa cell serum against thymus or bursa cells prepared from a 48-day-old line PDL-1 donor were examined. As shown in text-figure 3, both sera had specific cytotoxicity against the homologous cell type, but they had minimal reactivity against the heterologous cell type. Trypan blue dye barely stained dead thymus cells when antibursa cell serum dilution was less than 1:24; therefore, those data are not shown.

Table 1 shows the effect of treatment of PEC from an MDV-infected chicken (135 days old) with a specific antithymus or antibursa cell serum plus guinea pig complement. Every 2 days, 1 ml medium was added instead of the medium being changed. Results indicate that treatment of PEC with either the antithymus or antibursa cell serum plus complement did not remove the inhibitory effect of PEC from an MDV-infected chicken.

Influence of Addition of Sera on MDV Plaque Inhibition by PEC

Text-figure 4 shows the results of MDV plaque inhibition by normal PEC plus antiserum. PEC (1×10^6) from 3 normal chickens (85, 133, and 133 days old) were added to MDV-infected CKC cultures. To these cultures were added three different concentrations of sera from 3 MDV-infected PDL-1 chickens (AGP titer; ×2, ×2, and ×4) or sera from 3 normal chickens (negative for AGP antibody) (67-206 days old) to produce final concentrations of 10^{-1}, 10^{-2}, and 10^{-3}. Results show that normal PEC combined with serum from MDV-infected chickens inhibited MDV plaque

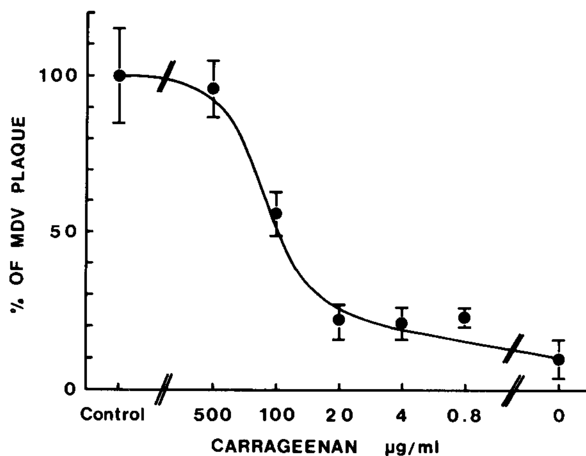


TEXT-FIGURE 3.—Specificities of antithymus and antibursa cell sera.

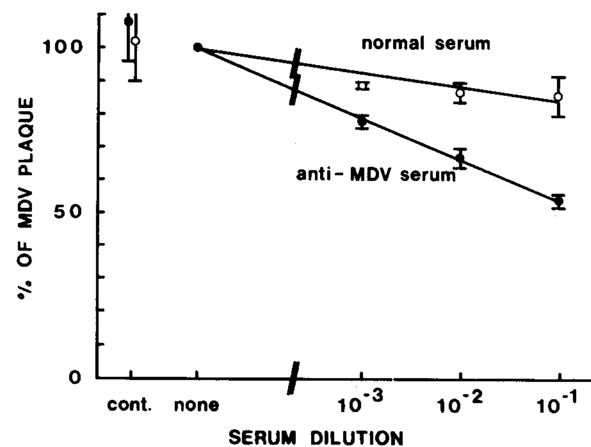
TABLE 1.—*MDV plaque inhibition by PEC treated with antithymus or antibursa cell*

PEC source	Treatment	Percentage of mean±SD[a]
MDV-infected chicken	Antithymus gland cell serum+ complement	8.6±7.4
MDV-infected chicken	Antibursa cell serum+complement	12.9±10.5
MDV-infected chicken	Complement	9.7±12.3
Control	None	100.0±22.0

[a] Mean value and SD are from 4 plates.



TEXT-FIGURE 2.—Effect of treatment of PEC with carrageenan on MDV plaque inhibition. *Each point and vertical line* represent the mean value and SD for three plates. Points above "Control" and "0" for carrageenan concentration are for PEC untreated control cultures and for cultures with PEC treated with medium, respectively.



TEXT-FIGURE 4.—Influence of addition of different concentrations of sera on MDV plaque inhibition by normal PEC. *Each point and vertical line* represent the mean value and SE for three tests. Points above "cont." and "none" for serum dilution are for cultures untreated with PEC and serum and for cultures treated with PEC only, respectively.

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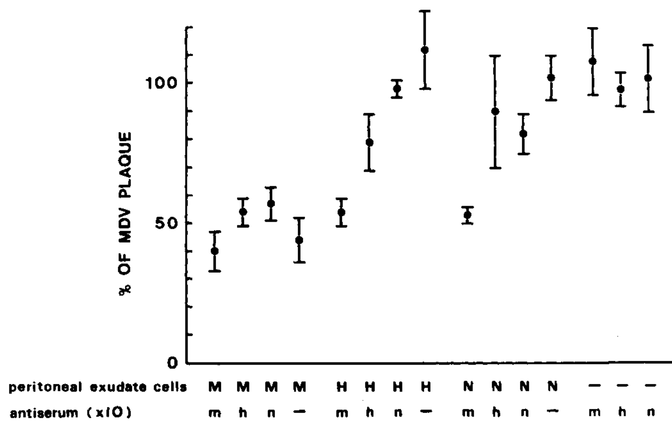

TEXT-FIGURE 5.—Influence of addition of sera from MDV-infected, HVT-vaccinated, or normal chicken on MDV plaque inhibition by normal PEC. *Symbols* represent PEC from chickens infected with MDV (M) or vaccinated with HVT (H) or from normal (N) chickens. Sera from chickens infected with MDV (m), vaccinated with HVT (h), or from normal chickens (n) were added at final concentrations of 10^{-1}. *Each point and vertical line* represent the mean value and SE for four PEC preparations.

formation, and the maximum inhibition was noted at a serum concentration of 10^{-1}. The inhibitory effect was statistically significant when compared to PEC with or without normal chicken serum ($P<0.01$ at 10^{-1} and 10^{-2}; $P<0.02$ at 10^{-3}).

Text-figure 5 shows the MDV plaque inhibition by PEC plus antiserum at a concentration of 10^{-1}. Two chickens each from MDV-infected chickens, HVT-vaccinated chickens, and normal chickens of the same age (85 and 93 days old) were used to prepare PEC in two separate experiments. Four sera from each of 3 groups of chickens were collected from 69- to 206-day-old birds. Sera from MDV-infected chickens or HVT-vaccinated chickens had titers of ×1 to ×4 as determined by the AGP test. To the MDV-infected CKC cultures were added 1×10^6 PEC and 10% serum from each group of chickens. Results show that when the PEC from HVT-vaccinated chickens and normal chickens were combined with serum from MDV-infected chickens, MDV plaque formation was inhibited. The results were statistically significant when compared to the PEC with no antiserum ($P<0.001$). In contrast, no marked inhibitory effect of PEC with the serum from HVT-vaccinated or normal chickens was observed.

DISCUSSION

The role of the reticuloendothelial system in immunity of MDV infection and tumor formation is not fully understood. Higgins and Calnek (*26*) examined the effect of silica treatment on newly hatched chicks and suggested that the spread of MDV seems to be restricted by the proliferation of macrophages. Lee et al. (*24*) showed that spleen macrophages of MDV-infected chickens suppress the stimulation of spleen lymphocytes by phytohemagglutinin. In the present experiment, we investigated antiviral immunity as related to macrophages. The data showed that PEC from an MDV-infected chicken inhibited MDV plaque formation and that this inhibition was removable by treatment of the PEC with carrageenan but not by treatment with antithymus or antibursa cell serum. The inhibitory effect of PEC was retained despite the medium's being changed every 2 days. These results indicate that MDV plaque formation was suppressed by macrophages.

Several mechanisms can be considered by which macrophages mediate a protective effect against MDV. The most probable mechanism is that macrophages react with the viral antigen expressed on the surfaces of the infected cells. Inasmuch as cell surface antigen induced by MDV can be detected a few hours after MDV infections of the cultured cells by the immunofluorescent technique (*27, 28*), effector cells in PEC may affect the infected cells at an early stage of virus infection before the virus has propagated and spread among the cells. Therefore, relatively few PEC from MDV-infected birds (10^5 or 3×10^5 cells/plate; text-fig. 1) might be able to inhibit MDV plaque formation. A second probable mechanism is that activated lymphocytes release lymphokines that alter macrophage function (*29*). This mechanism may occur in MDV plaque inhibition, but attempts to examine the suppressive effect with the products of cultured cells or cell extracts will be required. A third probable defense mechanism is that macrophages phagocytize and inactivate infectious cell-free virus. This mechanism cannot be applicable, however, to MDV, because infectious cell-free MDV is not produced in the cultures (*30*) and the virus is thought to spread between cells by cell-to-cell infection (*10, 31*).

Other factors may also influence macrophages to inhibit MDV plaque formation. Macrophages induced by irritants are activated and show cytopathic activity against syngeneic or allogeneic cells (*32*). In the present study, MDV plaque inhibition with more than 10^6 cells/plate observed by normal PEC might be caused by this mechanism. In addition, a cytotoxic effect against histocompatibility antigen of cultured cells may be involved because chickens used in this experiment are not inbred.

In antiviral T-cell-mediated immunity in MD, Ross (*18*) supposed that the inhibition of MDV plaque formation in CKC is due to direct lethal interaction between the sensitized lymphocytes and MDV-infected target cells. In the present study, PEC preparations contained 14±6% lymphocytes. Direct killing of target cells by MDV-sensitized lymphocytes may occur primarily at high concentrations of PEC.

The MDV plaque inhibition of PEC from HVT-vaccinated chickens and normal chickens was observed by addition of serum from MDV-infected chickens. This reaction may be ADCC mediated by killer cells present in the PEC preparation. It would be interesting to know why serum from HVT-vaccinated chickens is not effective in ADCC expression against MDV-infected cells in spite of the fact that a substantial antigenic relationship exists between MDV and HVT (*33*). The

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activity of ADCC has also been recognized in PBL against MDV-infected CKC (*17*). The ADCC reaction may be effective in the host because it can be provoked with a very small amount of antibodies. Firm identity of the cytotoxic effector cells, however, remains to be established.

In the present study, the macrophages seem to have a role in the suppression of viral replication in infected cells or in destruction of the cells by direct contact, thereby diminishing the chance of the spread of virus to adjacent susceptible cells. From the results of this study and from other reports (*17, 18*), the supposition is that antiviral immunity in MD is contributed by the cytotoxic effect of lymphocytes and macrophages, and in ADCC antiviral immunity is contributed by killer cells present in PBL and PEC.

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