

INTERFERON PRODUCTION BY MOUSE LEUKOCYTES IN VITRO AND IN VIVO

By LOWELL A. GLASGOW,* M.D., AND KARL HABEL, M.D.

(From the Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda)

PLATE 1

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The role of leukocytes in viral infections has never been fully defined. Early workers in the field demonstrated that viruses were not phagocytosed and destroyed in a fashion analogous to that of bacteria (1-7). In a number of studies virus has been found to be associated with white cells. Smith (6) demonstrated the presence of vaccinia virus in the leukocytes of rabbits after virus could no longer be isolated from whole blood. Sabin (5) confirmed the "fixation" of vaccinia virus by leukocytes *in vitro*, and interpreted his data to indicate that white cells were not viricidal for vaccinia. Other workers have failed to demonstrate inactivation of certain viruses by leukocytes *in vitro* (5, 8, 13), and in recent reviews of the work from his laboratory, Smorodintsev (9, 10) concluded that it demonstrated "the inactivity of phagocytic defense factors in all virus infections."

Some workers have postulated that viruses may be disseminated *via* leukocytes following observations that: (a) some viruses replicate in white cells (8, 11, 12), and (b) virus may be recovered in an infectious form from leukocytes of infected animals (1, 3, 6, 13, 14).

In contrast, more recent studies by several groups (13-16) have demonstrated adsorption and penetration or phagocytosis, but no multiplication of influenza virus in leukocytes. Ginsberg and Blackmon (13) investigated the rate of adsorption of PR-8 and Lee strains of influenza virus to guinea pig leukocytes. They were able to show that little or no virus eluted spontaneously and that less than 1 per cent of virus which did not elute could be demonstrated on disruption of the cells. Inglot and Davenport (14) confirmed these findings and, in addition, demonstrated that during a 20-hour period of observation: (a) leukocyte-associated virus, recoverable by disruption of cells, gradually decreased, and (b) influenza virus did not multiply in leukocytes.

Nishimi and Bernkopf (17) observed a similar phenomenon in mouse leukocytes infected *in vitro* with vaccinia virus. Virus titers in the supernatant fluids decreased, and they obtained no evidence of virus multiplication during the 96-hour period studied.

On the other hand, Florman and Enders (8) have reported multiplication of vaccinia virus to low titers in rabbit mononuclear cells cultured *in vitro*. More recently Bang and Warwick (18) and Goodman and Koprowski (19) have investigated virus

* Present address: Department of Pediatrics, University of Rochester, School of Medicine, Rochester 20, New York.

replication in macrophage cultures. Mouse hepatitis (18) was observed to multiply in macrophages grown from liver explants of genetically susceptible mice, but not in cultures from a resistant strain. Similarly, resistance of peritoneal macrophage cultures to group B arbor viruses reflected the natural resistance of the mouse strain from which they were derived (19).

The role of interferon in recovery from viral infections has recently been reviewed by Baron and Isaacs (20). Production of interferon has been associated with recovery from *in vivo* infections with O'nyong-nyong (21) and influenza viruses in mice (22). We have described two *in vitro* models in which interferon appeared to contribute to the elimination of a viral infection from tissue culture (23, 24). Gresser (25) first reported evidence which associated interferon production with white blood cells, and suggested that in this manner leukocytes could contribute to the host's defense mechanism.

The present study was initiated to investigate: (a) the course of vaccinia virus infection of leukocytes; (b) the production of interferon by virus infected leukocytes, and (c) the contribution of leukocyte-produced interferon to the host's defense mechanism.

Materials and Methods

Virus.—A calf lymph strain of vaccinia virus maintained by passage in monkey kidney cultures was obtained from Dr. Joel Warren. The virus pool was prepared in HeLa cells and titered 5×10^7 plaque-forming units (pfu) per ml.

Encephalomyocarditis virus (EMC) used in interferon assays was a large plaque-forming mutant, EMC r, obtained from Dr. K. K. Takemoto, National Institutes of Health. Virus pools were prepared in L cells and titered approximately 2×10^7 pfu per ml.

Vesicular Stomatitis virus, Indiana strain, (VSV) was obtained from the American Type Culture Collection. Virus pools were grown in ME-29, an established mouse embryo tissue culture line and titered 6×10^6 pfu per ml in primary mouse embryo cell cultures.

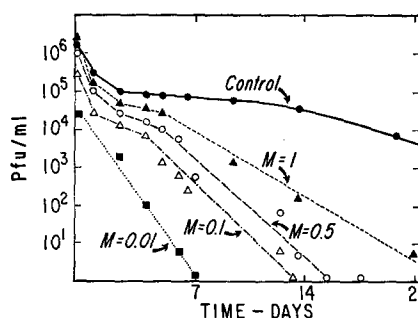
Chikungunya virus, standard reference strain, was obtained from Dr. Phillip Russell, Walter Reed Army Medical Center, Arbor Virus Unit, as 168th mouse brain passage. Virus pools were prepared by intracerebral (i.c.) inoculation of suckling, random-bred Swiss mice and titered approximately 2×10^7 pfu/ml.

Cells.—Primary mouse embryo cells used in interferon assays were obtained by trypsinization of 13- to 15-day embryos from random-bred Swiss mice. Leukocytes were obtained from 35 to 45 gm random-bred Swiss or inbred BALB/c mice. Donor animals were inoculated intraperitoneally (i.p.) with 1 to 2 ml of trypticase soy broth. White cell preparations containing 1 to 3×10^6 cells per ml were obtained 18 to 24 hours later by flushing the peritoneal cavity with 8 to 10 ml of Eagle's medium containing 1 per cent fetal calf serum. Differential counts of the peritoneal exudate carried out on Wright's stained preparations showed 42 to 68 per cent polymorphonuclear and 32 to 58 per cent mononuclear cells. Leukocyte cultures were maintained in 60 mm plastic petri dishes (Falcon Plastics, Los Angeles) in Eagle's medium with 15 per cent calf serum at 36° in a 4 per cent CO_2 atmosphere.

Virus Titrations.—Vaccinia virus was assayed by plaque method in HeLa cells. Virus was adsorbed for 3 hours and overlaid with 5 ml. of agar medium. A second overlay containing 1:20,000 neutral red was made on the 4th or 5th day and plaques counted on the 5th through the 8th day. Agar medium used for plaque assay contained 0.9 per cent Noble agar in Earle's BSS with 0.1 per cent bovine albumin and 0.1 per cent yeast extract.

Interferon Assay.—Culture fluids to be assayed for interferon were acidified with 6 N HCl to pH 2 and held for 22 to 24 hours. The pH was then adjusted to 7.2–7.4 with 5 N NaOH. Interferon preparations were free of infectious virus on inoculation of HeLa cultures. Interferon activity was assayed by the plaque reduction method. Monolayers of primary mouse embryo cells in plastic petri dishes were pretreated for 2 to 6 hours with 0.5 ml of the interferon or control preparations. Fluids were then removed and plates inoculated with approximately 50 pfu of EMC r virus. After 1 hour's adsorption, plates were overlaid with 5.0 ml of agar medium and incubated at 36°C. in the CO₂ incubator. Plates were stained with neutral red, final concentration of 1:30,000 at 24 hours and plaques counted at 28 and 48 hours.

Interferon.—Control interferon was prepared by infecting an established mouse cell line, 3-B (23), with 4×10^8 pfu/ml of Chikungunya virus and harvesting the supernatant at 2 days. Preparations were freed of virus as described above.



TEXT-FIG. 1. Course of vaccinia virus infection with varying input multiplicities (M : 1, 0.5, 0.1, 0.01) in cultures of mouse leukocytes. Virus assays are plotted as pfu/ml. The control curve represents the thermal inactivation of vaccinia virus in medium alone and in medium containing heat-killed leukocytes.

Inactivated virus.—Vaccinia virus (5×10^7 pfu/ml) was inactivated in a Habel-Sockrider ultraviolet apparatus as previously described (23).

Hemagglutinin Assay.—The technique described in "Diagnostic Procedures for Virus and Rickettsial Diseases" was followed (26).

Vaccinia Hyperimmune Serum.—Vaccinia antiserum was made in rabbits. A 1:20 dilution of serum incubated for $\frac{1}{2}$ hour at 36° with 5×10^7 pfu of vaccinia virus neutralized 99.8 per cent of the infectivity.

RESULTS

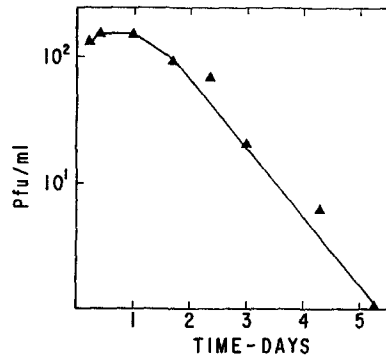
In Vitro Interferon Production by Leukocytes

Lack of Viral Multiplication in Mouse Leukocytes.—Initial studies were carried out to determine the course of vaccinia virus infection in mouse leukocytes *in vitro*.

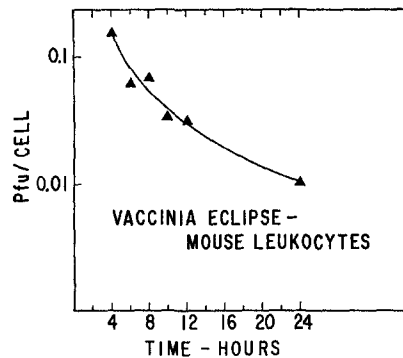
Replicate cultures of white cells, harvested from the peritoneal cavity as described, were inoculated with varying multiplicities of vaccinia virus. Samples of supernate were harvested periodically and assayed for virus.

At 48 to 72 hours after infection a cytotoxic effect was noted in cultures inoculated with a multiplicity of 0.5–1.0, but not in those receiving less than

0.5 pfu/cell. (Figs. 1 and 2.) This cytotoxic effect appeared similar to that described by Nishimi and Bernkopf (17) and was characterized by increased granularity and vacuolization with rounding and clumping of cells. Virus assays from this experiment are presented in Text-fig. 1. No evidence of virus



TEXT-FIG. 2. Vaccinia virus assays in mouse leukocytes inoculated with a multiplicity of 1.0. The inoculum was removed following 4 hours' adsorption. Virus titers represent pfu/ml in supernatant fluid.



TEXT-FIG. 3. Assays of intracellular vaccinia virus in mouse leukocytes. Vaccinia virus titers are expressed in pfu/cell.

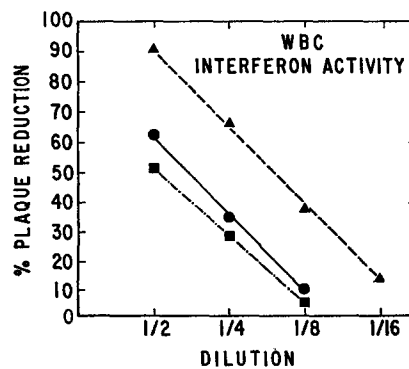
multiplication was obtained, virus titers gradually decreased, and after 7 to 21 days, depending on the input multiplicity of the inoculum, the vaccinia virus could no longer be detected in the supernatant fluids. The rate of inactivation was compared with a thermal inactivation curve of vaccinia virus in basal Eagle's medium with 15 per cent calf serum under similar incubation conditions and with that in replicate cultures of leukocytes inactivated by heating at 56° for 20 minutes. Thermal inactivation curves under both conditions were essentially identical and are presented in Text-fig. 1 as the control curve.

Vaccinia virus was eliminated from the leukocyte cultures at a rate which was significantly more rapid than that in the two thermal inactivation controls. These data suggest that the viable leukocytes were contributing to the elimination process and that minimal or no virus multiplication had taken place.

Because of the possibility that a limited replication of virus was being masked by the remaining large initial inoculum, a multiple cycle growth curve of vaccinia virus was obtained with the initial inoculum removed after virus adsorption had taken place.

TABLE I
Lack of Hemagglutinin Production in a Leukocyte Culture after Inoculation with Vaccinia Virus at a Multiplicity of 1.0 pfu/cell

Time of harvest, hrs.....	0	3½	7	11½	30	48	72
Hemagglutinin titer.....	256	64	0	0	0	0	0



TEXT-FIG. 4. Interferon activity, quantitated by per cent plaque reduction, in supernatant fluids of vaccinia infected cultures of mouse leukocytes (▲—▲) primary mouse embryo (●—●) and ME-29, an established mouse cell line (■—■).

Replicate cultures of mouse leukocytes were inoculated with an input multiplicity of 1.0. After a 4 hour adsorption, the inoculum was removed, cultures were washed three times, incubated at 36°, and samples of supernate collected for virus assay.

Results presented in Text-fig. 2 again indicate a lack of virus multiplication. The initial plateau in the curve of Text-fig. 2 is thought to represent elution of virus from cells following the washing procedure, although we have no direct evidence to substantiate this explanation.

Vaccinia Eclipse in Mouse Leukocytes.—The next experiment was designed to determine if vaccinia virus penetrated or was phagocytosed by leukocytes and to follow the fate of any intracellular virus.

Replicate cultures of leukocytes were inoculated with a multiplicity of 1.0 pfu/cell. After 3½ hours' adsorption the inoculum was removed, cultures were washed 3 times with Eagle's medium, and then treated with a 1:15 dilution of vaccinia hyperimmune rabbit serum for ½ hour. The hyperimmune serum was removed, cultures again washed 3 times and incubated at 37°. At varying time intervals individual cultures were harvested, cells disrupted by sonication and virus assayed.

Data from this experiment are presented in Text-fig. 3. Intracellular, non-neutralized virus decreased over the 24-hour period studied and there was no evidence of the synthesis of new infectious particles. This suggests that vaccinia virus entered, or at least became associated with leukocytes so that it was non-neutralizable by extracellular antiserum, and subsequently was converted to a

TABLE II
Identity of Characteristics of Leukocyte-Produced Interferon with Other Known Interferons

	Leukocyte Interferon	Control Interferon
Stability pH 2	+	+
Sediment 100,000 g	-	-
Species specificity	+	+
Active against heterologous virus	+	+
Stability at heating 60° for 60 min.	+	+
Stability at heating 80° for 60 min.	-	-
Trypsin-sensitive	+	+

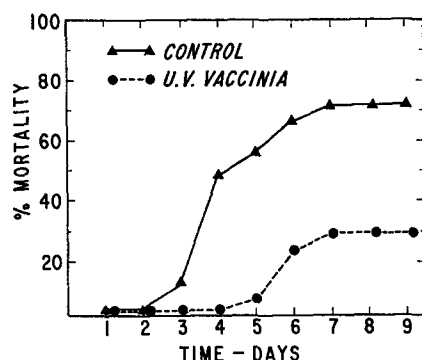
non-infectious form. This apparent intracellular eclipse could account for the more rapid rate at which infectious virus was lost from the leukocyte cultures as compared with controls in the previous experiment.

Lack of Hemagglutinin Production.—Henle, *et al.* (27) and Smith and Morgan (28) reported that influenza virus in HeLa and L cells produced a cytotoxic effect with little or no production of new infectious virus similar to that observed in our vaccinia-leukocyte system. In both influenza systems, however, virus hemagglutinin was produced in high titer. During the course of the previous experiment, samples of supernatant fluid were harvested and assayed for vaccinia hemagglutinin activity. Results of these assays are presented in Table I. Hemagglutinin decreased during the 3½-hour period of adsorption and no new hemagglutinin was produced during the course of the experiment. Thus vaccinia virus infection in mouse leukocytes was characterized by a cytotoxic effect which was not associated with the synthesis of new infectious particles or the release of viral hemagglutinin.

Interferon production by leukocytes.—

Cultures of leukocytes were inoculated with vaccinia virus at a multiplicity of 0.5 to 0.75. Supernatant fluids were harvested after 48 to 72 hours' incubation and assayed for interferon activity.

In Text-fig. 4, the plaque reduction obtained with serial dilutions of this harvest is compared to that produced by similar dilutions of fluids harvested from primary mouse embryo and ME-29 (23) cultures infected with vaccinia virus. In view of a greater cell population in leukocyte cultures these data should not be interpreted as demonstrating greater efficiency of interferon production by leukocytes on an individual cell basis. Per cent plaque reduction is calculated in relation to the pfu in control plates treated with dilutions of processed spent medium from non-infected leukocyte cultures. These results demonstrate that vaccinia virus-inoculated leukocyte cultures produce significant levels of an interferon-like substance. The properties of this substance are



TEXT-FIG. 5. Protective effect against VSV as the result of treatment designed to stimulate interferon production in peritoneal leukocytes of mice.

shown in Table II to be identical with those previously reported for other interferons.

In Vivo Effect of Leukocyte-Produced Interferon

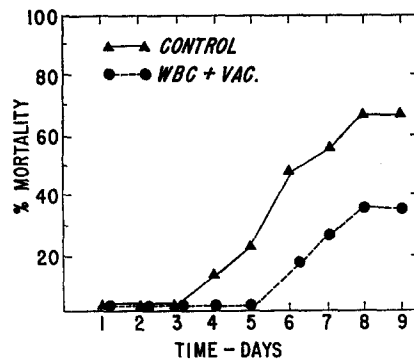
Protection against Vesicular Stomatitis Virus Challenge.—In view of the above evidence that mouse leukocytes can produce interferon after exposure to vaccinia virus *in vitro*, studies were initiated to determine if white cell produced interferon could contribute to the host's defense against virus infection *in vivo*.

Mice were inoculated intraperitoneally with trypticase soy broth to stimulate a leukocytic exudate. 24 hours later the test group received a 1.0 ml intraperitoneal inoculation of ultra-violet-inactivated vaccinia virus. After an additional 24 hours, mice were challenged intracerebrally with VSV. The control and test groups each consisted of 3 groups of 10 mice inoculated with approximately 50, 5, and 0.5 LD₅₀ of VSV.

The pooled cumulative mortality of these groups is illustrated in Text-fig. 5. There was a significant degree of protection in the groups receiving the inactivated vaccinia as manifested both by a delay in time of onset of symptoms

and by a reduced final mortality. Differences between control and test groups were significant at the 1 per cent level using the Kolmogorov-Smirnov test.

Ultraviolet inactivated virus was used because it is known to induce interferon production (23), yet no virus multiplication and minimal spread of inactivated virus particles to the CNS would be expected. By these means we hoped to minimize the possibility of direct viral interference affecting the course of the subsequent virus challenge. Samples of peritoneal exudate, blood and brain, collected 48 hours after inoculation, were assayed and found to contain no infectious virus.



TEXT-FIG. 6. Protective effect on VSV infection in mice by transfer of leukocytes stimulated to produce interferon *in vitro*.

Interferon Production by Leukocytes Exposed to Inactivated Vaccinia Virus in Vivo.—

Twenty-four to forty-eight hours after animals in the previous experiment were inoculated with the inactivated vaccinia virus the leukocytic peritoneal exudate was harvested from one group of test animals and from control mice which had received trypticase soy broth, but no inactivated vaccinia virus. Cells from both groups were maintained in tissue culture as previously described. After 48 to 72 hours supernatant fluids were harvested, pH-treated, and assayed for interferon activity.

Preparations from the cultures of leukocytes exposed to ultraviolet inactivated vaccinia virus *in vivo* effected a 50–79 per cent reduction in plaques in comparison with the control preparations. These results demonstrate that leukocytes exposed to virus *in vivo* are capable of producing interferon and are consistent with the hypothesis that leukocyte-produced interferon contributed to the observed protection against the VSV challenge.

Protection of Mice against VSV Challenge by Transfer of Leukocytes Exposed to Inactivated Vaccinia Virus in Vitro.—In order to localize the protective effect demonstrated in the previous experiment to the leukocytes and to relate it directly to the production of interferon by those cells, an experiment was de-

signed where isologous leukocytes, exposed to inactivated vaccinia virus *in vitro*, were transferred to normal mice. Recipient and control animals were then challenged with VSV inoculated intracerebrally.

Leukocytic exudate stimulated in the peritoneal cavity of inbred BALB/c mice was harvested and incubated *in vitro* for 3 hours with ultraviolet inactivated vaccinia virus. In order to minimize residual virus the cells were then centrifuged at 800 RPM for 10 minutes, washed in Eagle's medium, recentrifuged, and finally resuspended in Eagle's medium. Control leukocyte preparations received identical handling with the exception of the exposure to inactivated vaccinia virus. Groups of 15 test and control BALB/c mice received a 1.0 ml inoculation intraperitoneally containing 4 to 7×10^6 virus-exposed or control leukocytes. 24 hours later both groups were challenged with approximately 5 LD₅₀ of VSV. On the 4th to 5th day after the initial white cell transfer a second identical cell transfer was carried out.

TABLE III
Interferon Activity of Leukocytes Exposed to UV-Inactivated Vaccinia Virus Prior to Their Transfer to Isologous Normal Mice

Preparation assayed	Plaques per plate	Average plaques per plate	Plaque reduction
			<i>per cent</i>
Supernatant from WBC's + UV vaccinia....	10, 9, 14	11	70
Supernatant from WBC control.....	32, 29, 39	33	11
Interferon control.....	7, 4, 6	6	84
Control—no treatment.....	39, 35, 36	37	—

Cumulative mortality curves from this experiment are presented in Text-fig. 6. Animals in the test group which had received virus-treated leukocytes were shown to manifest resistance to the subsequent VSV challenge in comparison to those receiving control cells. The results presented are representative of data obtained in 3 experiments, and the differences in mortality are significant at the 5 to 10 per cent level.

Interferon Produced by Transferred Leukocytes.—The preceding experiment suggested that the virus-treated leukocytes were associated with the observed protection in recipient animals. The possible role of interferon, however, remained to be defined.

Samples of control and inactivated virus-treated leukocytes used in the transfer experiment were incubated *in vitro*. After 48 to 72 hours supernatant fluids were harvested, acid-treated as described, and assayed for interferon activity.

Results from one such experiment presented in Table III demonstrate that the transferred leukocytes did produce interferon.

DISCUSSION

It is apparent from numerous previous investigations that viruses are not destroyed by leukocytes as rapidly or as efficiently as is the case with many

bacterial pathogens. Any contribution of leukocytes to the host's defense mechanism in viral infections, however, has remained undefined. A number of viruses have been shown not to multiply in white cells and many of those that do replicate appear to achieve relatively low titers. We have considered the question of what function, if any, do leukocytes have in the host's defense against viruses. Recently published experimental results have strongly suggested that interferon may play an important role in the ability of the host to recover from virus infections. The association of white cells with interferon demonstrated in the present studies permits one to postulate a possible function of leukocytes.

Many viruses are taken up by leukocytes *in vivo*, but fail to multiply or do so relatively inefficiently. The result of this virus-cell interaction would be the production of interferon. This interferon would then be available to prevent extension of the virus infection to new susceptible cells and thus contribute to the elimination of the virus. The ability of leukocytes to accumulate at the local site of infection would enhance the effectiveness of the interferon produced by these cells. Although the evidence presented here establishes the capacity of white cells to produce interferon, quantitative data necessary to define the relative contribution of leukocytes to the defense of the host is still lacking.

Gresser and Enders (29) have recently demonstrated that a small population of resistant, interferon-producing cells in a culture of susceptible cells may alter the course of a virus infection in that culture. Our results suggest that white cells or reticuloendothelial system macrophages may perform a similar function *in vivo*. The observed persistence of virus particles in leukocytes may actually contribute to the recovery process by effecting a continued stimulation of interferon production. The present study tends to support such a hypothesis. Vaccinia virus has been observed to persist in white cells *in vivo* by Smith (6) and *in vitro* by Sabin (5) and in the present study. No new infectious virus was formed, but leukocytes so infected were shown to produce significant levels of interferon *in vitro*. The data also suggest that leukocytes produced interferon *in vivo* and that leukocyte-produced interferon contributed to the observed attenuation of the course of a VSV infection in mice.

SUMMARY

Vaccinia virus penetrates, or is phagocytosed by, mouse leukocytes *in vitro*. A cytotoxic effect is observed, but no new infectious virus is produced. Vaccinia virus, as infectious particles, is eliminated from a culture of leukocytes at a more rapid rate than can be accounted for by thermal inactivation. Leukocytes infected with vaccinia virus produce a substance with the properties of interferon. The evidence presented suggests that leukocytes also produce interferon *in vivo* and that this interferon is related to the observed protective effect on the outcome of intracerebral vesicular stomatitis virus challenge in mice. It is

postulated that leukocytes, in this manner, may make a positive contribution to the host's defense mechanism and to the process of recovery from viral infections.

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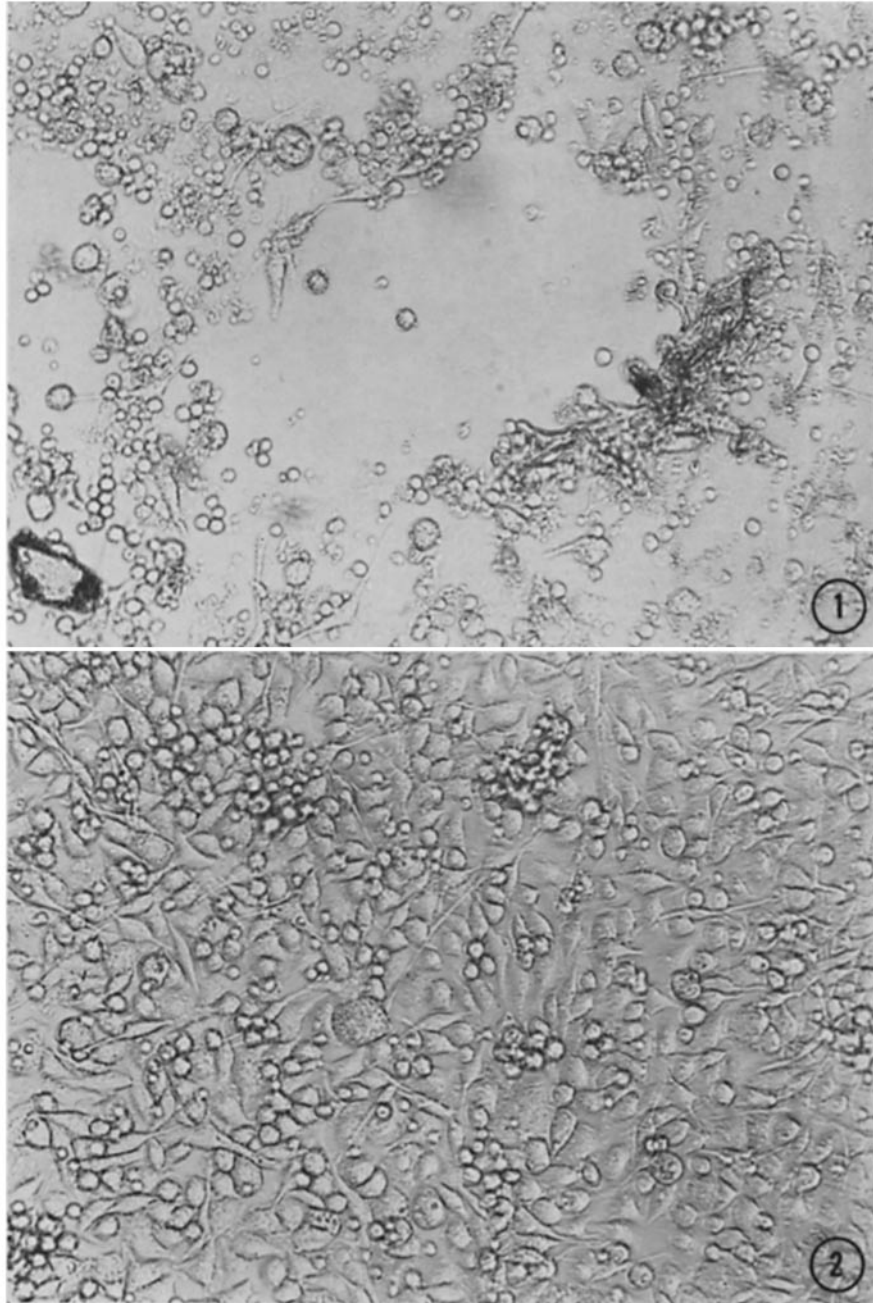
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EXPLANATION OF PLATE 1

FIG. 1. Cytotoxic effect of vaccinia virus on mouse leukocytes inoculated with a multiplicity of 1.0 and cultured for 48 to 72 hours. \times 430.

FIG. 2. Absence of obvious cytotoxic effect in a leukocyte culture inoculated with 0.1 pfu/cell of vaccinia virus and cultured for 48 to 72 hours. \times 430



(Glasgow and Habel: Interferon production by mouse leukocytes)