RABBIT MACROPHAGE INTERFERONS

I. Conditions for Biosynthesis by Virus-Infected and Uninfected Cells*

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The interferons are a biologically related but physically heterogeneous class of antiviral proteins which are produced by animal cells in response to viral infection or other inducing agents (1).

The primary factors which appear to determine the physical properties and molecular weight of each interferon are the animal species of the producing cell (2) and the nature of the inducing agent (3, 4). Moreover, there is also suggestive evidence that different tissues of the same animal may produce different interferons in response to the same stimulus (5). Of particular interest from the standpoint of pathogenesis of viral infection is the role of phagocytic cells in interferon production (6).

Among the many questions raised by these observations is whether there are different mechanisms or different genes that control biosynthesis of different interferons. Previous studies have clearly demonstrated that virus-induced formation of interferons by cultures of chick (7, 8) and mouse (8) fibroblasts or cancer cells (9) requires intact capacity to synthesize cellular RNA and protein. However, inhibitors of protein synthesis were not found to prevent interferon formation induced in mice by intravenous injection of bacterial endotoxin (10) or the *Penicillium* mold product, statolon (11). The failure heretofore to demonstrate endotoxin-induced formation of interferon in cell cultures has precluded a more exacting comparison of interferon induction by viruses and bacterial endotoxin.

The present studies were undertaken to define more precisely the mechanisms of synthesis and the nature of interferons produced by parenchymal and phagocytic cells. Explanted rabbit cells and intact rabbits were chosen as experimental models because of their capacity to synthesize large amounts of interferon at an extremely rapid rate in response to viral and nonviral stimuli. The first paper describes the kinetics and cellular mechanisms of interferon synthesis under various conditions. The following report (12) compares some physical properties and molecular weights of the several interferons.

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Materials and Methods

Media.—Growth medium (GM)[1] for primary cultivation of cells consisted of 0.65% lactalbumin hydrolysate (LAH) in Earle's buffered salt solution (BSS) (Grand Island Biological Co., Grand Island, N. Y.), 5% calf serum (CaS), and antibiotics (penicillin, 200 units/ml; streptomycin, 200 μg/ml). Maintenance medium (MM) refers to LAH supplemented with 2% CaS. Solid medium for plaque assay contained 1% agar in LAH and 2% CaS.

Viruses.—The CG strain of Newcastle disease virus (NDV) was grown in the allantoic sac of chick embryos infected at 12 days of age. After incubation at 39°C for 36 hr, infected allantoic fluid was harvested, pooled, clarified by low-speed centrifugation, and stored at −60°C until use. This stock NDV suspension had a hemagglutinin titer of 256 when tested with 0.5% chicken erythrocytes, and 1 ml contained 3.2×10^8 plaque-forming units (PFU) as assayed by plating on monolayers of chick embryo cells. The Indiana strain of vesicular stomatitis virus (VSV) was grown in monolayer cultures of primary chick embryo cells (13).

Glycogen.—Shellfish glycogen (Mann Research Laboratories, New York, N. Y.) was prepared as a 0.1% solution in nonpyrogenic physiologic saline containing penicillin, 125 units/ml, and streptomycin, 400 μg/ml.

Endotoxin.—Lipopolysaccharide extracted from *Escherichia coli* strains 0127:B8 and 026:B6 (Difco Laboratories, Detroit, Mich.) was dissolved in LAH at a concentration of 1000 μg/ml.

Inhibitors of Macromolecular Synthesis.—Actinomycin D was a gift of Dr. F. D. Lawrason (Merck, Sharp & Dohme, West Point, Pa.). Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

Cultivation of Rabbit Kidney Cells.—Primary cultures of rabbit kidney (RK) cells were prepared by trypsinization of minced kidney tissue of 3-wk-old rabbits. Plastic Petri plates (diameter, 60 mm) were seeded with 3×10^6 washed cells suspended in 5 ml of GM; milk-dilution bottles (surface $\simeq$40 cm^2) were seeded with the same number of cells suspended in 10 ml of GM. All cultures were incubated at 37°C in an atmosphere of ~5% CO_2. Confluent monolayers formed in 5 days with only one change of medium required.

Collection and Cultivation of Leukocytes.—Sterile peritonitis was induced in 6–8 lb New Zealand white rabbits by intraperitoneal infusion of 350–400 ml of glycogen solution (14); cells were harvested either 18 or 72 hr later. When rabbits were sacrificed at 18 hr, 100–200 ml of leukocyte-rich exudate (~10^7 cells/ml) could be aspirated from the peritoneal cavity. After chilling the harvested exudates, the cells were sedimented by centrifugation at 1500 rpm for 10 min at 4°C and resuspended to the desired concentration in cold GM. Approximately 95% of the cells in these early exudates were polymorphonuclear (PMN) leukocytes.

Cells were also harvested 72 hr after glycogen infusion to obtain a population consisting mainly of macrophages. Since very little fluid was present in the peritoneal cavity at this time, exudate was obtained by washing twice with 100 ml of cold GM containing 0.5% heparin. Cells were sedimented as described above and resuspended in smaller volumes of cold GM. The average yield of cells per rabbit at 72 hr was 5×10^7, about 90% of which were mononuclear cells and the remainder PMN leukocytes.

Alveolar macrophages were obtained by the method described by Myrvik et al. (15).

Cultures of PMN or mononuclear leukocytes were prepared by seeding milk-dilution bottles with cells suspended in 10 ml of GM. Leukocytes readily attached to the glass when

[1] The following abbreviations are used in this paper: GM, growth medium; LAH, 0.65% lactalbumin hydrolysate; BSS, Earle's buffered salt solution; CaS, calf serum; MM, maintenance medium, LAH supplemented with 2% CaS; VSV, vesicular stomatitis virus; RK, rabbit kidney; PMN, polymorphonuclear leukocytes; PDD, plaque-depressing dose.

stationary cultures were incubated at 37°C but they did so less readily at 4°C. In most experiments interferon production was induced in cultures that contained 2–8 × 10^7 leukocytes. PMN leukocytes were also tested at much higher concentrations.

Interferon Assay.—The plaque inhibition method, as described in a previous communication (8), was modified as follows: Cell-free media from NDV-infected cultures were subjected to three 1-hr cycles of centrifugation at 100,000 *g*, dialyzed overnight against HCl at pH 2, and redialyzed to neutrality against BSS. Media from uninfected cultures to be tested for interferon content were not centrifuged or dialyzed. Individual fluids were then diluted serially in 2-fold steps in MM; 2 ml aliquots of each dilution were adsorbed on duplicate, drained cultures of RK cells and incubated at 37°C for 24 hr. The cultures were then drained, infected with 50–100 PFU of VSV, overlayered with agar-containing media, and incubated again at 37°C for 2 days. Plaques were scored after staining the cell sheets with neutral red, 1:1000. Interferon titers are expressed as the reciprocal of the dilution that depressed the plaque count to 50% of that in control cultures (PDD_{50}/ml).

RESULTS

Virus-Induced Interferon Synthesis

Comparative Cellular Responses to NDV Infection.—These initial experiments were designed to determine the rate and amount of interferon synthesis by RK cells and peritoneal leukocytes.

Bottle cultures of RK cells, macrophages, or PMN leukocytes were infected with 1 ml of stock NDV. Virus was allowed to adsorb for 30 min at 37°C, following which the RK cells were covered with 9 ml of MM and the leukocytes with 9 ml of GM; the higher serum content of GM appeared to afford better preservation of leukocytes. Interferon yields were not influenced by washing the cell layers after the period of virus attachment. At intervals after incubation at 37°C, the medium was completely aspirated from each culture, processed for interferon assay, and replaced with fresh medium.

The representative experiments shown in Fig. 1 demonstrate the similarity in rate of interferon synthesis after NDV infection of the three types of cell cultures. In each case, no interferon could be detected at 1 hr but was invariably present by 2 hr after virus adsorption, and maximal synthesis occurred between 2 and 6 hr. Titers at 24 hr were approximately twice those at 6 hr. When the interferon titers were plotted on an arithmetic scale, the rate of production in all three systems was seen to be linear between 2 and 6 hr. These data suggest similar kinetics of virus-induced interferon synthesis for the competent cells in each type of culture.

No valid comparisons can be made of the relative efficiency of interferon synthesis by RK cells and macrophages. The lower yield from RK cells may well be due to the smaller total cell population, which was approximately 10% of that in the macrophage cultures. However, the probability exists that PMN leukocytes are not the principal interferon-producing cells in cultures prepared from 18-hr peritoneal exudates. Although Fig. 1 shows that large quantities of interferon were produced in these cultures, approximately 5% of the 8.8 × 10^8 cells were mononuclear leukocytes. In comparison, even higher yields of inter-

feron were produced by 8×10^7 cells from 72-hr exudates, of which 90% were macrophages and the rest contaminating PMN leukocytes. Further studies revealed that macrophages invariably produced larger quantities of interferon than did equal numbers of PMN leukocytes. Owing to the unavailability of pure cell cultures, it was not possible to determine whether PMN leukocytes

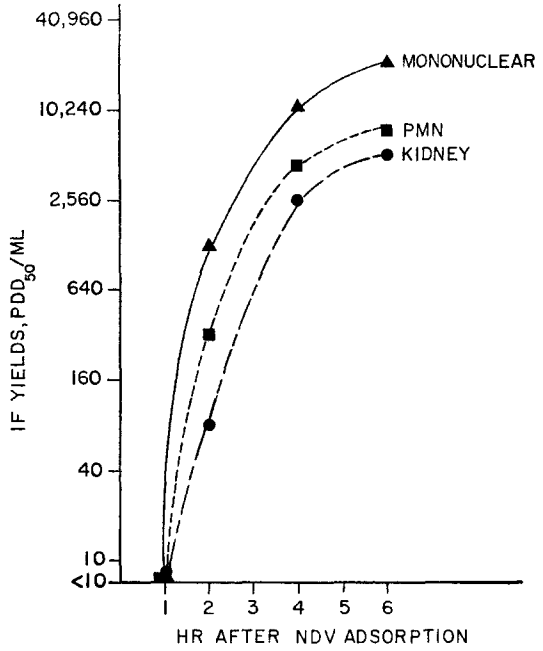

FIG. 1. Interferon (IF) synthesis by rabbit cell cultures infected with 3.2×10^8 PFU of NDV. The approximate numbers of cells in each culture were: mononuclear leukocytes from 72-hr peritoneal exudates = 8×10^7 cells (90% macrophages, 10% PMN leukocytes); PMN leukocytes from 18-hr peritoneal exudates = 8.8×10^8 cells (95% PMN, 5% mononuclear); kidney (RK culture) = 6×10^6 cells.

were competent to synthesize any interferon. In general, interferon yields from cultures of peritoneal leukocytes were a function of the number of macrophages.

The capacity of cultured spleen cells to synthesize interferon was also determined in a single experiment. Minced splenic tissue from a rabbit that had been infused intraperitoneally with glycogen solution 3 days previously was trypsinized, and the dispersed cells (predominantly mononuclears) were seeded in culture bottles.

Infection of these cells with NDV and incubation at 37°C for 20 hr resulted in an interferon yield of 10,240 PDD_{50}/ml.

Effect of Actinomycin on NDV-Induced Synthesis of Interferon by RK Cells

and Macrophages.—One way to compare the interferon-synthesizing efficiency of RK cells and macrophages is to determine by indirect means the rate at which each cell type transcribes interferon-specific messenger RNA. In previous studies with Krebs-2 mouse carcinoma cells (9) it had been shown that inhibition of cellular RNA synthesis by actinomycin prevents interferon formation provided that the antibiotic is introduced into cell cultures within 4 hr after

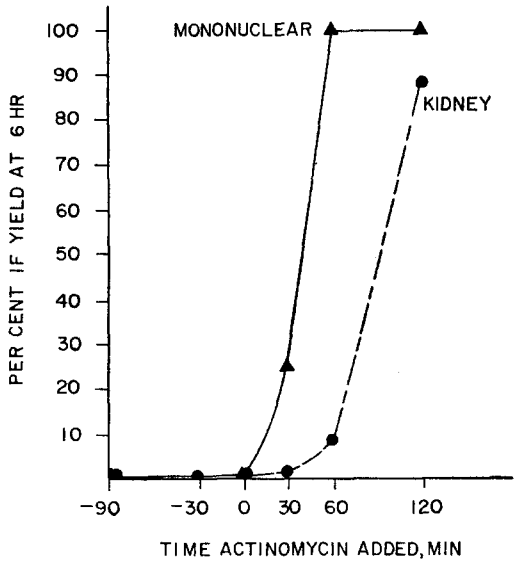

FIG. 2. Effect of actinomycin (10 μg/ml) on 6-hr yields of NDV-induced interferon (IF) from cultures of primary RK cells (kidney) and peritoneal macrophages (mononuclear). Actinomycin was added to the medium before, during, or after NDV adsorption (time 0), and allowed to remain in contact with the cell layers for 60 min (−90 to −30, −30 to +30, 0 to +60, +30 to +90, +60 to +120, or +120 to +180 min). Per cent interferon yields from actinomycin-treated cultures are based on titers in replicate cultures not exposed to actinomycin.

NDV infection. After this time, actinomycin has no effect on continuing interferon synthesis, a finding which suggests that mRNA is transcribed early and, once transcribed, its translational function is stable. Based on the preceding experiments which demonstrated earlier and more rapid interferon production by NDV-induced rabbit cells, the prediction could be made that interferon-specific mRNA is transcribed sooner in rabbit cells than in mouse (9) or chick (8) cells.

At intervals before or after NDV infection, actinomycin was added to the media in a series of RK or macrophage cultures to give a final concentration of 10 μg/ml. After exposure to the antibiotic for 1 hr, the actinomycin-containing media in each culture were completely re-

moved, the cell layers were washed, and the cultures replenished with fresh media without actinomycin. Parallel control cultures infected with NDV were not exposed to actinomycin but were washed and the media replaced in the same manner. The media in all control and actinomycin-treated cultures were removed at 6 hr after NDV infection and assayed for interferon content.

The results of these experiments, shown in Fig. 2, are expressed as per cent final yields of interferon from actinomycin-treated cultures based on the 6-hr yields from each matching control culture. As noted, little or no interferon

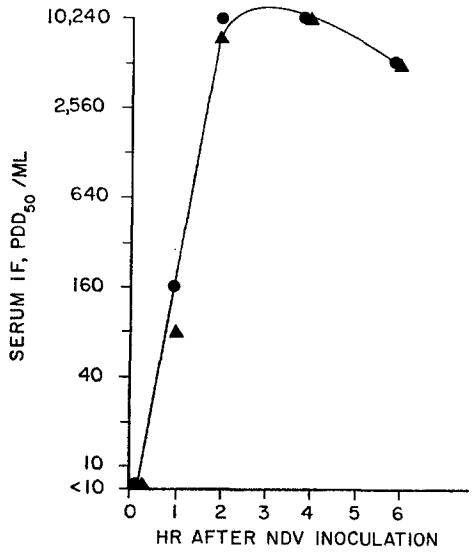

FIG. 3. Titers of interferon (IF) in serum of two rabbits at intervals after intravenous injection of 3.2×10^8 PFU of NDV. Each symbol represents a different rabbit.

(<5-30 PDD_{50}/ml) was produced by RK cells or macrophages exposed to actinomycin for 1 hr prior to or simultaneous with viral infection. The titers in corresponding control cultures ranged from 1920 to 10,240 PDD_{50}/ml. When actinomycin was present in the medium from 30 to 90 min after virus adsorption, the 6-hr yield from RK cells was reduced to 1.5% and from macrophages only to 25%. Actinomycin added to cultures at 1 or 2 hr had no effect on the 6-hr interferon yield from macrophages. In contrast, RK cells treated with the antibiotic at 1 hr synthesized only 8% of the expected final yield. By 2 hr after NDV infection, the interferon-synthesizing capacity of RK cells was not significantly influenced (88% of control yield) by actinomycin.

These data suggest that transcription of the interferon-specific mRNA of rabbit macrophages begins almost immediately after viral infection and is com-

pleted between 30 and 60 min, whereas the transcriptive event in RK cells begins later and requires approximately 120 min to reach completion.

Comparative Interferon Response to Intravenous Injection of NDV.—One question raised by the preceding experiments is whether macrophages and related cells are a primary source of interferon produced in rabbits in response to intravenous injection of NDV. Baron and Buckler (16) had originally shown prompt appearance of circulating interferon in mice injected intravenously with NDV and other viruses. Kono and Ho (17) extended these observations by demonstrating that rabbits respond even better and suggested that the source of interferon is cells of the reticuloendothelial system. Thorotrast diminishes the interferon response (17) and tolerance can be induced by prior injection of virus or bacterial endotoxin (18, 19). It was of interest, therefore, to compare NDV-induced interferon synthesis in intact rabbits with that of cultured rabbit macrophages.

1 ml of stock NDV was injected into the marginal ear vein of rabbits which were bled from the central ear artery at intervals thereafter. Sera from these rabbits were subjected to acid dialysis (pH 2 for 24 hr), neutralized, and assayed for interferon.

The representative experiment shown in Fig. 3 demonstrates that interferon appeared in the circulation even more rapidly than it did in macrophage cultures exposed to comparable amounts of virus. Interferon was readily detected within 1 hr after intravenous injection of NDV, reached a peak of 10,000 PDD_{50} /ml or greater by 2-4 hr, and declined thereafter. By 48 hr the serum titers fell below the threshold of detectability. There was no evidence of persistent viremia; serum obtained at 2, 4, and 6 hr was free of hemagglutinin and plaque-forming virus.

The existence of tolerance to a second intravenous injection of virus (18) was readily confirmed. The mean 6-hr interferon titer in serum of rabbits injected intravenously for the first time with 1 ml of NDV was 5120 PDD_{50}/ml, whereas 6-hr serum interferon titers in the same animals given a second intravenous injection 48 hr later were only 20–160 PDD_{50}/ml.

An experiment was then performed to measure the capacity of peritoneal macrophages obtained from tolerant rabbits to synthesize interferon in vitro.

Sterile peritonitis was induced by glycogen infusion in 10 rabbits and 24 hr later 5 of these were injected intravenously with 1 ml of NDV. Peritoneal leukocytes were harvested from all animals 72 hr after glycogen infusion (48 hr after intravenous injection of NDV) and individual cultures established with the cells obtained from each rabbit.

Table I shows the 6-hr interferon yields from each of these cultures after infection in vitro with 1 ml of NDV. Although the total cell counts and proportion of macrophages ($\sim$ 90%) were similar in each group of cultures, less interferon was produced by macrophages from animals injected 48 hr previously with NDV. The mean interferon titer in 6 control cultures was 10,240 PDD_{50}/ml

compared with a mean of 1653 PDD_{50}/ml in cultures of macrophages from tolerant rabbits. Although this represents a reduction of 85% in interferon yield, the relative unresponsiveness to NDV of macrophages from tolerant rabbits does not account completely for the far greater reduction (~99%) in amount of circulating interferon in similar rabbits challenged intravenously with NDV.

TABLE I

Comparative Yields of NDV-Induced Interferon from Cultures of Peritoneal Macrophages Harvested from Control and Tolerant Rabbits

Cell source	Rabbit No.	Cells/culture	6-hr IF yield
		$\times 10^7$	*PDD 50/ml*
Control* rabbits	1‡	4.4	20,480
	1‡	4.4	10,240
	2	3.3	10,240
	3	1.1	10,240
	4	5.8	7,680
	5	2.0	2,560
Mean		3.5	10,240
Tolerant* rabbits	6	3.7	2,560
	7	3.4	2,560
	8	3.0	1,920
	9‡	5.9	960
	9‡	5.9	960
	10	1.7	960
Mean		3.9	1,653

* Peritonitis was induced in all ten rabbits by infusion of glycogen solution; 24 hr later rabbits 6–10 were made tolerant by injection intravenously with 1 ml of stock NDV. Rabbits 1–5 served as controls. Macrophage cultures were established from 72-hr peritoneal exudates, infected with NDV, and interferon titers assayed 6 hr later. Note the lack of correlation between cell number and interferon yield.

‡ Two cultures prepared from the same rabbit.

Interferon Synthesis by Uninfected Macrophages

"Spontaneous" Production of Interferon.—It was of interest to determine whether cultured macrophages can release preformed interferon or synthesize interferon *de novo* in the absence of viral infection.

For this purpose cultures were established from macrophage-rich exudates harvested from the peritoneal cavity of rabbits 72 hr after glycogen infusion. In one series of experiments the cells were incubated at 37°C immediately after seeding the cultures, and the media assayed for interferon content at intervals thereafter. In other experiments the harvested cells were seeded in the same manner, but the cultures were first kept at 4°C for 24–48 hr prior to incu-

bating them at 37°C. Culture fluids to be assayed for interferon activity were freed of cells and gross cellular debris by centrifugation at 1500 rpm but not subjected to ultracentrifugation or acid dialysis.

Fig. 4 demonstrates that prior exposure to virus is not essential for production of interferon by peritoneal macrophages. Although the average yields were only 1% of those from comparable cultures of NDV-infected macrophages, the rates

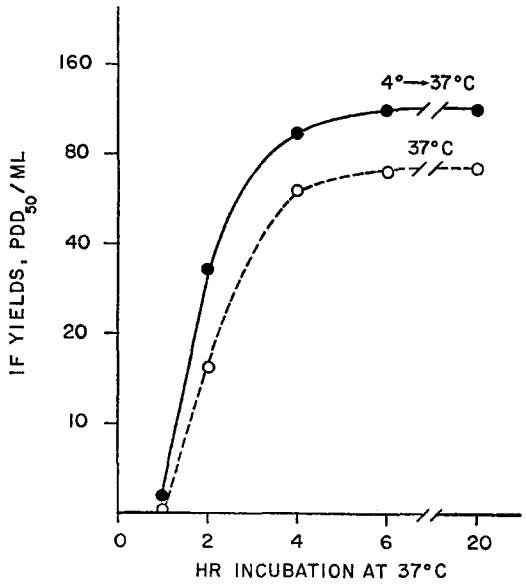

FIG. 4. Interferon (IF) synthesis in the absence of viral infection in cultures of 3.7×10^7 rabbit peritoneal macrophages. One set of cultures (37°C) was incubated at 37°C directly after seeding cells harvested from 72-hr peritoneal exudates. The replicate set of cultures (4° → 37°) was kept at 4°C for 48 hr and then transferred to 37°C (time 0).

of interferon synthesis were similar (compare with Fig. 1). Interferon activity could be readily detected in uninfected cultures of 3.7×10^7 cells between 1 and 2 hr after incubation at 37°C and reached a peak at 4–6 hr. No interferon was released by uninfected macrophages incubated at 4°C for as long as 48 hr. However, when prechilled cultures were warmed to 37°C, the yields of interferon were slightly but consistently higher than the yields from macrophages incubated directly at 37°C (Fig. 4). The production of interferon by uninfected macrophages appears to represent active synthesis by viable cells. No interferon activity could be recovered from freshly harvested cells disrupted by ultrasonic vibration, nor was interferon produced when cell lysates were incubated in GM at 37°C for 6 hr.

Of the cell types studied, this capacity to produce interferon "spontaneously"

appears to be restricted to macrophages. Repeated tests were made of media from uninfected cultures prepared from 18-hr peritoneal exudates rich in PMN leukocytes; these cells produced no interferon or did so only in barely detectable amounts, which could be attributed to contaminating mononuclear cells. In addition, uninfected spleen cells or RK cells failed to release measurable amounts of interferon during incubation of cultures at 37°C for 20 hr.

Two experiments were performed to determine whether the glycogen used to stimulate the formation of peritoneal exudates was also responsible for inducing interferon synthesis. In one, sterile peritonitis was produced by intraperitoneal infusion of fine glass beads suspended in pyrogen-free saline. Exudates harvested 72 hr later were similar in number and proportion of macrophages to the exudates obtained 72 hr after glycogen infusion. Incubation of 6.6×10^7 cells in 10 ml of medium at 37°C resulted in rapid appearance of interferon, which reached a peak titer of 160 PDD_{50}/ml at 4–6 hr. In another experiment, an attempt was made to obtain mononuclear cells from rabbits without peritonitis. The peritoneal cavities of seven normal rabbits washed with large volumes of GM yielded a total of 2.8×10^7 cells, 80% of which were large mononuclears. These cells were seeded in a single bottle and the medium sampled at intervals after incubation at 37°C. At 4 and 6 hr the interferon titer was 5 PDD_{50}/ml but none could be detected in earlier or later samples.

The result of this experiment suggests that mobilized macrophages in peritoneal exudates have a greater capacity to produce interferon than do mononuclear cells normally present in the peritoneum.

The following experiment was designed to determine whether uninfected macrophages that are actively producing interferon can also synthesize virus-induced interferon.

Two replicate cultures of peritoneal macrophages obtained 72 hr after glycogen infusion were kept at 4°C overnight and then warmed to 37°C. One culture was infected with NDV immediately upon warming, at which time no interferon was present in the medium, and the other was infected with NDV 4 hr after incubation at 37°C, when the medium contained 80 PDD_{50}/ml of interferon.

By 6 hr after viral infection, the interferon titers of the media from both cultures rose to 5120 PDD_{50}/ml. Thus, active interferon production by uninfected macrophages had no effect on their ability to synthesize virus-induced interferon.

These experiments demonstrate that uninfected macrophages have the intrinsic capability of producing interferon without overt stimulus, as well as in response to viral induction, but there is no way to ascertain whether the same cells produce both kinds of interferon.

Interferon Production by Macrophages Induced by Bacterial Endotoxin.—In view of the findings by other investigators (3, 4) that intravenous injection of bacterial endotoxin results in rapid appearance of circulating interferon, macrophage cultures were tested for their capacity to respond to the same inducing agent.

E. coli lipopolysaccharide was added to the media in freshly planted cultures of peritoneal macrophages; culture fluids were sampled at intervals after incubation at 37°C and assayed for interferon content. The fluids were not processed by ultracentrifugation or acid dialysis, nor were any attempts made to rid them of residual endotoxin prior to testing for interferon.

Endotoxin had no direct effect on VSV plaque formation, nor did it stimulate interferon production in RK cells; incubation of RK cells in 2 ml of media con-

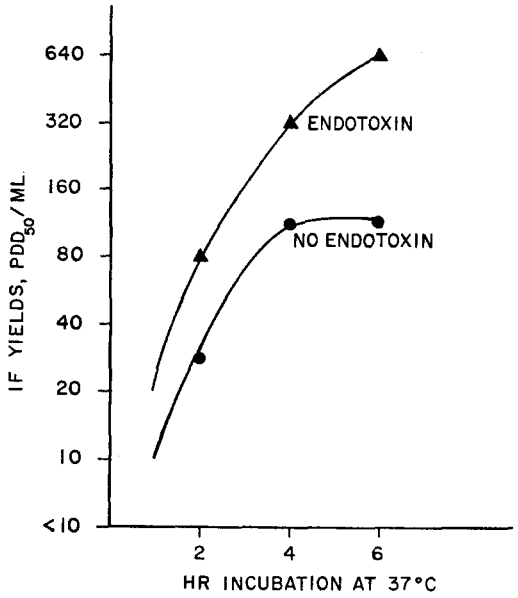

FIG. 5. Enhanced synthesis of interferon (IF) by 4.5×10^7 uninfected macrophages incubated at 37°C in the presence of *E. coli* endotoxin, 100 μg/ml.

taining 1000 μg of endotoxin for 24 hr prior to VSV challenge resulted in no significant reduction in plaque numbers.

Fig. 5 compares the rate and extent of interferon production by 4.5×10^7 rabbit peritoneal macrophages incubated at 37°C in media with or without endotoxin (100 μg/ml). The 6-hr interferon yields were generally 5- to 6-fold greater in cultures incubated in media containing endotoxin at concentrations of 100 or 10 μg/ml. Titers in macrophage cultures exposed to 1 μg/ml were not significantly greater than those in parallel cultures to which no endotoxin had been added. No interferon was produced by macrophages incubated at 4°C for 24 hr in media containing endotoxin at a concentration of 100 μg/ml. These data clearly indicate that *E. coli* endotoxin in high dosage can induce or augment interferon synthesis by peritoneal macrophages in the absence of viral infection.

The results of these experiments also raised the question whether "sponta-

neous" interferon synthesis was in fact due to induction by bacterial endotoxin contaminating cultures of macrophages. It was found that tissue culture media used in preceding experiments to harvest and cultivate leukocytes were invariably pyrogenic for rabbits, as was specially prepared media obtained from other suppliers. In addition, several batches of serum (calf and horse), which appear to be essential for maintaining leukocytes in culture, were found to induce typical endotoxin fevers when injected intravenously into rabbits. An attempt was also made to harvest and cultivate peritoneal macrophages in a pyrogen-free saline solution of 0.31% sodium lactate (Lactated Ringer's Injection, Cutter Laboratories, Berkeley, Calif.) with or without 5% rabbit serum and 0.1% glucose. No interferon was synthesized by macrophages incubated for 6 hr in these media or in 100% rabbit serum. The addition of endotoxin to the saline-lactate solution resulted in production by macrophages of low yields of interferon (20 PDD_{50}/ml) in 6 hr. The poor interferon responses of macrophages in cultures incubated under these conditions are probably attributable to cellular injury in a suboptimal environment. Therefore, no proof could be obtained that interferon is synthesized by uninfected peritoneal macrophages in the complete absence of bacterial endotoxin.

Peritoneal macrophages are not unique in their ability to synthesize endotoxin-induced interferon. Alveolar macrophages, washed from lungs of normal rabbits, were cultured at a concentration of 1.4×10^7 cells in 10 ml of GM containing 100 μg/ml of *E. coli* endotoxin. After incubation for 6 hr at 37°C, supernatant fluid contained interferon at a titer of 30 PDD_{50}/ml.

Effect of Actinomycin and Puromycin on Interferon Production by Uninfected Macrophages.—There would appear to be two alternative mechanisms by which interferon is produced by cultures of uninfected macrophages: (*a*) interferon could be synthesized *de novo* by transcription of mRNA followed by translational protein synthesis in a manner similar to virus-induced interferon formation (9); or (*b*) an interferon precursor could be activated or preformed interferon released from cells, as postulated by Youngner et al. (10) for endotoxin-induced interferon formation in intact mice.

These alternative hypotheses were tested by blocking cellular RNA synthesis with actinomycin and protein synthesis with puromycin at various stages of interferon production by macrophages cultivated at 37°C. The rationale behind these experiments is as follows: (*a*) if interferon is synthesized *de novo* by uninfected macrophages, then actinomycin should inhibit its formation only during an early transcriptive stage but not later, whereas puromycin should inhibit throughout the course of active interferon formation; (*b*) if no new cellular RNA or protein synthesis is required for interferon formation, neither actinomycin nor puromycin should have an appreciable effect at any stage.

Cultures of peritoneal macrophages which had been kept at 4°C for 24 hr were warmed to 37° and actinomycin (10 μg/ml) was added to the media in individual cultures immediately (time 0) or 30 or 60 min later. After exposure to the drug for 1 hr, media were removed from these and parallel control cultures not exposed to actinomycin; the cell layers were then

washed and covered with fresh medium without actinomycin. At 6 hr from the start of incubation at 37°C, media were collected from all cultures, dialyzed for 24 hr against two changes of Earle's saline solution to remove residual actinomycin, and assayed for interferon content. Final interferon yields at 6 hr from each actinomycin-treated culture were expressed as per cent of yield from the corresponding control culture.

Fig. 6 shows that interferon synthesis was completely inhibited by actinomycin added to macrophage cultures immediately upon warming (time 0) and

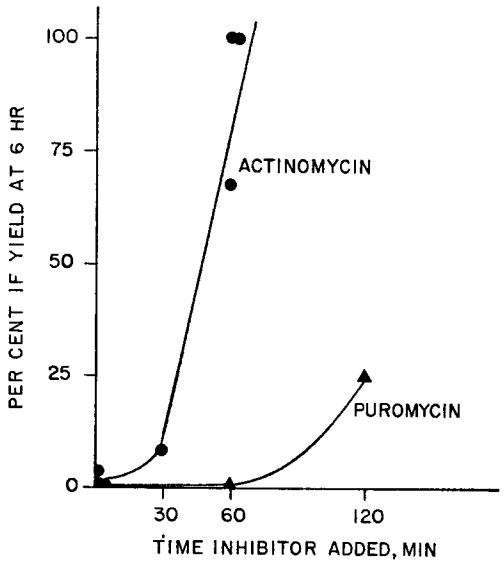

FIG. 6. Comparative effects of actinomycin (10 μg/ml) and puromycin (50 μg/ml) on 6-hr yields of interferon (IF) from uninfected macrophages. Each inhibitor was added to cultures at the time they were transferred from 4° to 37°C (time 0) or at intervals thereafter. Actinomycin was present in the medium for 60 min (0–60, 30–90, or 60–120 min), whereas puromycin remained in contact with the cells from the time that it was added to each culture. Per cent interferon yields were calculated from titers in matched control cultures not exposed to either inhibitor but incubated at 37°C for 6 hr.

the titer was reduced to 8% of control yields when the drug was added at 30 min. By 60 min the capacity of uninfected macrophages to produce interferon was completely or almost completely refractory to actinomycin; in three separate experiments the 6 hr interferon yields were 67, 100, and 100% of yields from control cultures which titered 160–240 PDD_{50}/ml.

The effect of puromycin on interferon production by uninfected macrophages was tested in the same manner except that puromycin (50 μg/ml) was present in the medium from 0, 60, or 120 min after warming the cultures to 37°C and remained in contact with the cells until 6 hr, when the experiment was terminated. At this time the media from puromycin-treated and control cultures were withdrawn, dialyzed, and assayed for interferon content.

Fig. 6 demonstrates that puromycin added to the medium at time 0 or 60 min completely blocked interferon production. When puromycin was introduced at 120 min, the final 6-hr yield was 60 PDD_{50}/ml compared with 240 PDD_{50}/ml in the corresponding control culture. As indicated in Fig. 4, uninfected macrophages can be expected to produce about 25% of their total interferon yield in 2 hr. These data show that puromycin, unlike actinomycin, shuts off interferon synthesis both early and late.

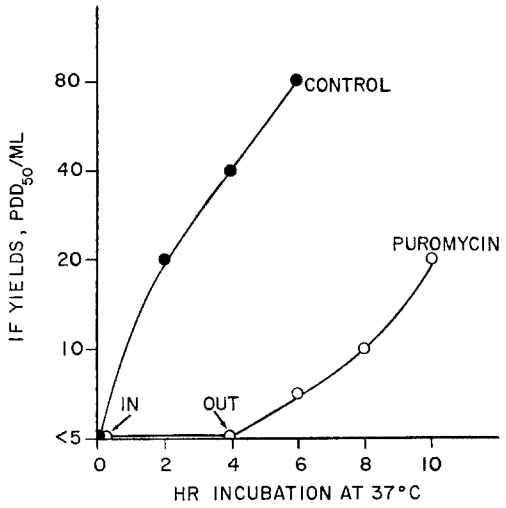

FIG. 7. The degree of reversibility of puromycin inhibition of interferon (IF) synthesis by uninfected macrophages (5.6×10^7 cells/culture). Puromycin (10 μg/ml) was added to the medium at the start of incubation at 37°C (time 0, IN). At 4 hr (OUT) the medium was drained off, the cell layers washed twice, and the cultures replenished with fresh medium without puromycin. Control cultures were never in contact with puromycin.

Fig. 7 demonstrates that inhibition of interferon synthesis by puromycin is partially reversible. In this experiment, macrophages were first incubated at 37°C for 4 hr in media containing puromycin (10 μg/ml); no interferon could be detected at this time. When the puromycin was removed and the cell layers were washed, interferon production resumed but the yields were considerably lower than those in control cultures. Failure to reverse the inhibitory effect completely may be attributable to residual puromycin or to poor cell survival.

Endotoxin-induced synthesis of interferon by macrophages was also inhibited by actinomycin (10 μg/ml) and puromycin (50 μg/ml). No interferon could be detected at 6 hr when either inhibitor was present in the medium from the time of exposure to 100 μg/ml of *E. coli* lipopolysaccharide, whereas interferon titers in parallel control cultures rose to 160–320 PDD_{50}/ml by 6 hr after exposure to endotoxin.

These data strongly suggest the existence of an induction mechanism leading to *de novo* synthesis of interferon-specific mRNA and interferon by uninfected macrophages similar to that for virus-induced synthesis of interferon.

DISCUSSION

The macrophage has long been considered a likely candidate as a cell that may play a signficant role in host defense against viral infection.

Mims (20) has reviewed the evidence that the pathogenesis of many viral infections depends to some extent on the capacity of macrophages to transport various viruses and to support viral replication at tissue sites of primary lodgment. The outcome of infection with mouse hepatitis virus, for example, appears to be genetically determined by differential viral replication in peritoneal macrophages of inbred strains of resistant and susceptible mice (21). The physiological basis for these observations has not been shown to be related to the phagocytic function of macrophages nor to direct virucidal activity of their lysosomal contents. Although the present studies, and others (6), provide only indirect evidence that macrophages in inflammatory exudates contribute to antiviral defenses of the host, these cells appear to be ideally adapted for such a role by virtue of their capacity to produce interferon rapidly and in large quantities. Moreover, the actinomycin experiments indicate that transcription of interferon-specific mRNA begins almost immediately after virus adsorption and is completed earlier than it is in rabbit kidney cells or parenchymal cells of other species (8, 9).

Peritoneal macrophages have been shown to originate from circulating mononuclear cells (22). Several investigators have demonstrated that human blood leukocytes can also produce interferon in response to viral infection (23, 24) or following exposure to phytohemagglutinin (25). In fact, human leukocytes can provide a rich source of interferon for potential therapeutic use.[2] Wheelock (26) has recently obtained convincing evidence that mononuclear cells (lymphocytes ?) rather than polymorphonuclear leukocytes are the competent interferon-producing cells in separated cellular fractions of human blood. Mouse peritoneal leukocytes, predominantly macrophages, also produce interferon in response to viral infection (27, 28) as do alveolar macrophages (29). Although far from conclusive, our findings are consistent with the foregoing evidence that the macrophage is the principal, if not the only, interferon-producing cell in rabbit peritoneal exudates. However, the best available data indicate that reticuloendothelial cells of the spleen, rather than circulating mononuclear cells, are the major source of interferon that appears in serum of animals soon after intravenous injection of virus. Van Rossum and De Somer (30) have found that interferon titers in spleen are considerably higher than titers in blood or other tissues of rats injected intravenously with NDV, and Fruitstone et al. (31) have demonstrated that splenectomy markedly reduces the levels of circulating interferon in mice. These findings help to explain some of the differences in interferon responses of cultured macrophages and intact rabbits as observed in our experiments.

The capacity of macrophages to produce interferon in the absence of viral induction

[2] Falcoff, E., R. Falcoff, F. Fournier, and C. Chany. Personal communication.

appears to be a unique property of these cells. Rabbit kidney cells and PMN leukocytes cultured from early peritoneal exudates did not exhibit this capability. Similar observations have been made very recently by Nagano et al.,[3] who demonstrated rapid "spontaneous" release of interferon by cultured macrophages obtained from rabbits with paraffin-induced peritonitis. It is of interest in our experiments that uninfected macrophages in 72-hr peritoneal exudates induced by glycogen or glass beads were far better producers of interferon than were macrophages normally present in the peritoneal cavity or lungs of rabbits without apparent inflammatory lesions. Cohn and Benson (32) have demonstrated that mononuclear phagocytes undergo differentiation during mobilization which results in changes in their morphology, cytochemistry, and biochemistry as compared with resting mononuclear phagocytes. Although our data permit only speculation on this point, it is conceivable that the capacity to synthesize interferon "spontaneously" is related to macrophage maturity and, hence, could be an integral part of the inflammatory process. A weakness in this hypothesis is the technical difficulty we encountered in excluding pyrogen from the media in our cultures of peritoneal macrophages. However, although enhanced production of interferon undoubtedly occurs in cultures of macrophages incubated in the presence of added endotoxin, extremely large quantities are required to increase interferon yields.

As is the case with virus-induced interferons, no conclusions can be drawn concerning the cellular origin of circulating interferon which appears shortly after intravenous injection of endotoxin (4, 33). The yield of endotoxin-induced interferon and its rate of production are similar in intact animals and macrophage cultures. However, one striking difference between the in vivo and in vitro systems is the failure of actinomycin to block interferon production in rabbits (34), and of puromycin or cycloheximide to block interferon production in mice (10), in response to intravenous injection of endotoxin. These findings led Ho and Kono (34) and Youngner et al. (10) to postulate the existence in animal tissues of preformed interferon, which is presumably released or activated by endotoxin. Our experiments, which conclusively demonstrate that actinomycin and puromycin switch off interferon synthesis by uninfected macrophages, do not necessarily refute the Ho-Youngner hypothesis as it applies to the intact animal, but the macrophage data clearly indicate conditions under which endotoxin can induce *de novo* synthesis of interferon or, alternatively, synthesis of an interferon precursor or activator.

Active interferon synthesis by uninfected macrophages was not found to alter their capacity to respond to viral induction with further interferon synthesis. The heterogeneity of the cell population precluded meaningful experiments to determine whether the same cells produce both interferons. It is apparent, however, that small amounts of interferon present in cultures of uninfected macrophages did not inhibit production of virus-induced interferon. Presumably, both interferons can be synthesized concurrently. Nor did we observe the priming effect of small amounts of interferon on subsequent virus-induced interferon synthesis, as reported recently by Freedman (35)

The present experiments revealed no evidence of different mechanisms or different genes which control interferon synthesis by uninfected and virus-

[3] Nagano, Y., Y. Kojima, J. Arakawa, and R. Kanashiro. Personal communication.

infected macrophages. Although virus-induced interferon is produced in much larger quantities, rates of synthesis are substantially the same, and the effects of inhibitors of RNA and protein synthesis are indistinguishable. Early transcriptive and later translational events appear to be required in both systems. Virus-induced and endotoxin-induced interferons produced by rabbit macrophages could be distinguished only by studies of their molecular weight, as described in the accompanying report (12).

SUMMARY

Interferon is produced in cultures of rabbit leukocytes in response to infection with Newcastle disease virus or in the absence of known viral infection. The macrophage appears to be the responsible producing cell. Cultures prepared from sterile peritoneal exudates, which contained about 90% macrophages, are at least as efficient as cultures of rabbit kidney (RK) cells in their capacity to synthesize NDV-induced interferon. Interferon can be detected in the medium by 2 hr after viral infection and the titers usually reach a peak of 10,000 PDD_{50}/ml by 4–6 hr. Exposure to actinomycin prior to or shortly after viral induction effectively blocks interferon synthesis by cells of both types. However, macrophages become refractory to actinomycin by 30–60 min compared with 60–120 min for RK cells, a finding which suggests earlier and more rapid transcription of interferon-specific messenger RNA in macrophages. Macrophages harvested from the peritioneal cavity of rabbits injected intravenously with NDV 48 hr previously also exhibit slightly reduced capacity to synthesize interferon, but this tolerant state is less marked than is tolerance to production of circulating interferon in intact rabbits.

Interferon is also synthesized by rabbit macrophages not infected with virus but simply incubated at 37°C in medium with or without added bacterial endotoxin. Uninfected polymorphonuclear leukocytes, rabbit kidney and spleen cells produced no detectable interferon under similar conditions of cultivation. No interferon was released by intact macrophages incubated at 4°C or by ultrasonically disrupted macrophages incubated at 37°C. Although interferon titers were found to be higher when uninfected cultures were exposed to 10–100 μg/ml of *E. coli* lipopolysaccharide, unavailability of suitable pyrogen-free maintenance media precluded answering the question whether macrophages can continually synthesize and release interferon spontaneously. Interferon yields from uninfected macrophages were only 1% or less of the yields from NDV-infected macrophages, but the rates of synthesis were similar under both conditions. Studies with actinomycin and puromycin revealed that sequential transcriptive and translational events are required for *de novo* interferon synthesis by uninfected cells in a manner similar to virus-induced interferon synthesis.

The physical properties and molecular weights of these rabbit interferons are discussed in the following report (12).

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