RABBIT MACROPHAGE INTERFERONS

II. Some Physicochemical Properties and Estimations of Molecular Weights*

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The experiments reported in the preceding paper (1) revealed essentially similar kinetics of interferon synthesis in primary cultures of rabbit macrophages and renal epithelium. Although interferon yields from uninfected macrophages were much lower than those from virus-infected cells, rates of synthesis were similar and actinomycin introduced soon after induction inhibited interferon production in all in vitro systems. The only significant difference in interferon-synthesizing capacity of virus-infected macrophages and kidney cells was the time after induction at which the respective cell types became refractory to the inhibitory action of actinomycin. None of these data can be interpreted as indicating that more than one molecular species of interferon is synthesized by different rabbit cells in response to the same or different inducing agents. However, other investigators have found that the stability and molecular weights of mouse, rabbit, and human interferons appear to depend on the producing cell type and the viral or nonviral nature of the inducing agents (2–4).[1]

The present report presents evidence that macrophages and other cells can produce several molecular species of interferon that are heterogeneous with respect to molecular weight despite their apparent similarity in biological and other physicochemical properties.

Materials and Methods

Sources of Interferons.—Details of the procedures were presented in the preceding report (1). Virus-induced interferons were prepared by harvesting media from primary cultures of 2–8 × 10^7 rabbit peritoneal macrophages or 2–4 × 10^6 rabbit kidney (RK)[2] cells that had

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[1] In addition: Falcoff, E., R. Falcoff, F. Fournier, and C. Chany. Personal communication.

[2] The following abbreviations are used in this paper: RK, rabbit kidney; VSV, vesicular stomatitis virus; PDD, plaque-depressing dose; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EA, ovalbumin; STI, soybean trypsin inhibitor.

been infected 4, 6, or 24 hr previously with Newcastle disease virus (NDV) at input multiplicities per cell of 5–100 plaque-forming units (PFU). Serum interferon was obtained from rabbits bled 4–6 hr after intravenous injection of 1 ml of stock NDV suspension which contained 3.2×10^8 PFU and 1024 hemagglutinin units. Residual virus was largely removed from media or serum by three 1-hr cycles of centrifugation at 100,000 *g* and, unless otherwise indicated, by dialysis for 24 hr against HCl buffer at pH 2. Endotoxin-induced interferon was produced by incubating cultures of uninfected macrophages for 6 hr at 37°C in media containing *Escherichia coli* lipopolysaccharide, 100 μg/ml. Endotoxin-induced interferon was clarified by low-speed centrifugation but not routinely subjected to ultracentrifugation or acid dialysis.

All unfractionated interferons were assayed by incubating 2 ml of serial 2-fold, or more closely spaced, dilutions for 24 hr with replicate cultures of RK cells, which were then challenged with 50–100 PFU of vesicular stomatitis virus (VSV). Interferon titers are expressed as the 50% plaque-depressing dose (PDD_{50}/ml).

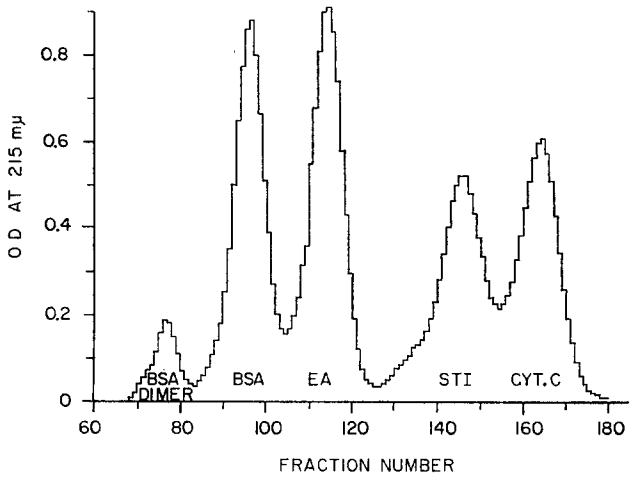

FIG. 1. Calibration of Sephadex G-100 column by elution of protein standards of known molecular weight. Optical density (OD) at wave length 215 mμ was determined for the following proteins: bovine serum albumin (BSA), ovalbumin (EA), soybean trypsin inhibitor (STI), and cytochrome *c* (CYT. C). Fractions collected were 4 ml each.

Sephadex Gel Filtration.—A procedure suitable for high resolution chromatography of interferons on Sephadex gels has been described in detail (5). A column of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) with a diameter of 3.35 cm and a bed height of 110.5 cm (bed volume = 974 ml) was equilibrated at 4°C with phosphate-buffered saline (PBS, pH 7.4) at a flow rate of 20 ml/hr. Samples of 2 ml each were carefully layered on top of the gel bed with the aid of a peristaltic pump and capillary plastic tubing. Effluent from the column was collected in 4-ml fractions by means of a volume-operated fraction collector.

The column was calibrated by the method of Andrews (6) with only minor modifications. The following marker proteins of known molecular weight were used as internal reference standards: bovine serum albumin (BSA), monomer mol wt ≃67,000 and dimer mol wt ≃134,000 (Armour Pharmaceutical Co., Kankakee, Ill.); ovalbumin (EA), mol wt ≃45,000

(Nutritional Biochemical Corp., Cleveland, Ohio); soybean trypsin inhibitor (STI), mol wt ≃21,500 (Worthington Biochemical Corp., Freehold, N. J.); and cytochrome *c*, mol wt ≃13,000 (Nutritional Biochemical Corp.). The void volume of the column was determined with dextran blue, mol wt ≃2×10^6 (Pharmacia, Uppsala, Sweden). Each of the proteins was dissolved in PBS to a concentration of 35 mg/ml, filtered through a 0.45 μ Millipore filter, and 0.1 ml (3.5 mg) was incorporated into the sample to be layered on the column. Protein concentrations in effluent fractions were determined by optical density in a Beckman DU spectrophotometer operated at a wave length of 215 mμ.

Fig. 1 shows the elution pattern of these proteins. The results were reproducible on repeated determinations and the optical density peaks did not vary more than one fraction. Recovery of marker proteins in the effluent ranged from an estimated 70 to 90% of the applied sample. Dextran blue was excluded from the gel and appeared in peak concentration at a volume of 276 ml (fraction 69).

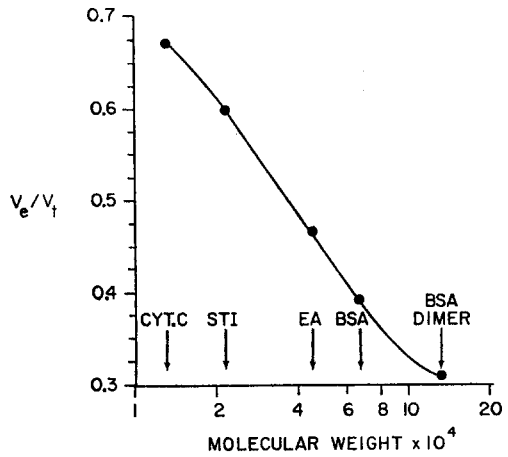

FIG. 2. Reference curve for estimation of molecular weights by Sephadex filtration. Peak elution volumes (V_e) divided by total column volume (V_t) are plotted against logarithm of known molecular weights of the protein standards shown in Fig. 1.

The reference point for the molecular weight of each protein standard was taken as the peak fraction and expressed as the elution volume (V_e) divided by the total volume (V_t) of the column. As shown in Fig. 2, when V_e/V_t for all four proteins was plotted against the logarithm of the known molecular weights of each protein (and BSA dimer), a linear function could be described over a molecular weight range from 21,500 (STI) to 67,000 (BSA monomer). The molecular weights of unknown proteins, such as interferons, can be estimated fairly precisely by comparing their V_e/V_t values to those of the marker proteins if they fall within the linear portion of the curve.

Molecular Weights of Interferons Estimated by Sephadex Filtration.—In these studies interferon preparations were filtered through Sephadex G-100 along with one or more marker proteins of known molecular weight. Interferon activity of effluent fractions was quantitated by the plaque-reduction method using VSV as test virus. Aliquots of two adjacent fractions were first pooled and assayed at low dilutions (usually 1:5 or 1:10) to bracket the zone or zones of interferon activity. Each active fraction (or fractions) was then tested on three to five monolayer cultures of RK cells at a dilution estimated to give 20–80% plaque inhibition.

Peak fractions were considered to be those with the highest interferon titer based on the greatest degree of plaque reduction at the dilution tested. These values were not converted to absolute PDD_{50} units of interferon because such data are not meaningful in the context of these experiments. Molecular weights of the various interferons were estimated by comparing V_e/V_t of peak interferon activity to that of marker protein peaks and were read directly from the plot of V_e/V_t vs. log molecular weight shown in Fig. 2.

The validity of estimating molecular weights of interferons by this method is primarily a function of the precision of the bioassay procedure. As previously shown by Lindenmann and Gifford (7) and reviewed by Wagner et al. (5), the sampling error in interferon assays can be considerably reduced by increasing the number of plates used for assay at each dilution.

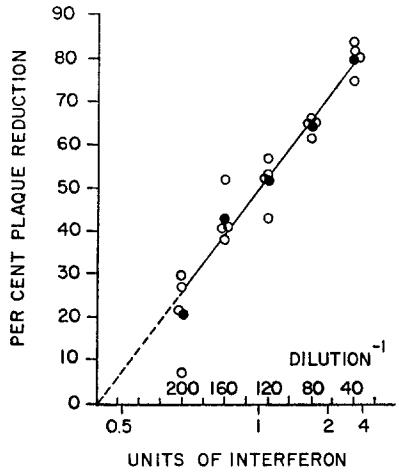

FIG. 3. Analysis of per cent reduction in VSV plaque formation plotted as a function of interferon dilution. A single fraction of column effluent obtained by Sephadex filtration of serum interferon was diluted in parallel and aliquots of each dilution tested on four RK cultures (open circles, individual plates; closed circles, means). Five control plates not exposed to interferon contained an average of 122 plaques. One unit of interferon is arbitrarily equated to 50% plaque reduction.

In addition, accuracy of the titration procedure can be enhanced by making parallel rather than serial dilutions.[3] Fig. 3 shows a representative test of the reliability of the method using five closely spaced parallel dilutions of an effluent fraction collected from the Sephadex column after filtration of serum interferon. As noted, a linear relationship obtained when interferon dilution was plotted against plaque reduction over a range of 20–80%. The use of three (or more) replicate plates at each dilution minimized the variability inherent in this assay. Therefore, a reasonably accurate estimate of interferon activity can be made by testing plaque inhibition at a single dilution of each fraction eluted from the Sephadex column.

RESULTS

Physicochemical and Biological Characterization

Criteria for Establishing Identity of Four Rabbit Interferons.—By definition (8), all interferons have certain properties in common regardless of their cellular

[3] Smith, T. J. Unpublished data.

and species origin or the nature of the inducing agent. These criteria for classification as interferons were met by each of the tissue culture preparations obtained from the following sources: uninfected macrophages incubated at 37°C without inducer, macrophages exposed to bacterial endotoxin, RK cells infected with NDV, and macrophages infected with NDV. The results of these experiments can be summarized as follows:

Trypsin sensitivity: 1-ml samples of each diluted interferon was incubated at 37°C with 100 μg of crystalline trypsin (Worthington Biochemical Corp.) and the reaction stopped at 30 min by adding 200 μg of soybean trypsin inhibitor. In each case original titers of 160 or 60 PDD_{50}/ml were reduced to 10 or <5 PDD_{50}/ml.

Nonsedimentability: Each preparation was centrifuged three times at 100,000 *g* for 1 hr. The supernatants showed no loss in activity.

Nondialyzability: Titers were unchanged after dialysis against 100 volumes of Earle's buffered salt solution (pH 7.4) for 24 hr at 4°C.

Species specificity: Monolayer cultures of chick embryo cells incubated for 24 hr in the presence of undiluted rabbit interferons were fully susceptible to plaque formation by VSV.

Absence of direct antiviral activity: VSV mixed with the respective interferons and incubated for 1 hr at room temperature showed no loss in plaque-forming titer.

Lack of virus specificity: Both NDV-induced and endotoxin-induced interferons protected RK cells against cytopathic effects of encephalomyocarditis virus.

By these criteria, the four rabbit interferons were identical regardless of cellular source or inducing agent.

Comparative Lability of Virus-Induced and Nonvirus-Induced Interferons.— Other investigators have reported that serum and leukocyte interferons induced by endotoxin (3) or phytohemagglutinin (4) are considerably more labile to heat and acid treatment than are virus-induced interferons produced by the same animal species. This distinguishing characteristic was found to hold only to a limited extent for rabbit interferons.

The four rabbit interferons described above were diluted to comparable titers in Earle's buffered saline solution and heated for 1 hr in a water bath maintained at 56°C or dialyzed at 4°C against 100 volumes of HCl buffer at pH 2 for 24 hr.

Table I shows that such treatment caused no appreciable reduction in titer of stock preparations of NDV-induced interferon obtained from RK or macrophage cultures. In contrast, exposure to heat or acid resulted in approximately 85% loss in activity of interferons prepared from uninfected macrophages. These differences in heat and acid lability were confirmed repeatedly. Nonvirus-induced interferons lost 75–90% of their antiviral activity regardless of initial titer, whereas virus-induced interferons were more stable.

It was also necessary to determine whether a labile component had been destroyed during the preliminary dialysis step used as routine by us, and almost all investigators, to rid virus-induced interferons of residual virus.

Accordingly, an interferon sample from a 6-hr culture of NDV-infected macrophages was freed of most of the residual virus only by three 1-hr cycles of centrifugation at 100,000 *g*.

Table II demonstrates that heating at 56°C or exposure to HCl resulted in 50% reduction in antiviral activity. Consecutive acid and heat treatment of the same sample caused a slight further drop in titer.

Thus, virus-induced macrophage interferon is not completely stable, as had been assumed, although it is significantly less labile than endotoxin-induced interferon synthesized by the same cell population. The possibility that residual

TABLE I

Heat and Acid Lability of Different Rabbit Interferons

Tissue culture source		Interferon titer after treatment		
Cell type	Inducer	Control	56°C, 1 hr	pH 2, 24 hr
		PDD_{50}/ml	PDD_{50}/ml	PDD_{50}/ml
Kidney	NDV	60	40	60
Macrophage	NDV	120	120	120
Macrophage	None	60	10	10
Macrophage	Endotoxin	320	40	40

TABLE II

Lability of NDV-Induced Rabbit Interferons

Treatment	Interferon titer and source	
	Macrophage	Serum
	PDD_{50}/ml	PDD_{50}/ml
Control	5,120	81,920
pH 2, 24 hr	2,560	20,480
56°C, 1 hr	2,560	20,480
pH 2, 24 hr + 56°C, 1 hr	1,920	15,360

virus in the supernatant after centrifugation might represent the labile component could be excluded on the following grounds: first, potent anti-NDV immune serum did not affect interfering activity; and second, undiluted NDV-induced rabbit macrophage interferon had no effect on plaque formation by VSV plated on cultures of chick embryo cells.

Data recorded in Table II also demonstrate significant lability of interferon present in rabbit serum 4 hr after intravenous injection of NDV. Repeated tests showed that serum interferon heated to 56°C for 1 hr or dialyzed against buffer at pH 2 for 24 hr lost 75–80% of its original activity. Heating to 56°C following exposure to acid resulted only in a slight further reduction in titer of serum interferon (Table II), a finding which suggests that the acid stable component is also, for the most part, heat stable.

None of the interfering activity in sera of rabbits injected intravenously with NDV could be attributed to residual circulating virus. No diminution in interfering activity occurred when serum interferon was ultracentrifuged or incubated with anti-NDV immune serum which could completely neutralize 3.2×10^8 PFU of NDV. Moreover, no plaque-forming or hemagglutinating virus could be recovered from sera of rabbits 2, 4, and 6 hr after intravenous injection of NDV, and undiluted sera with interferon activity of 80,000 PDD_{50}/ml were completely incapable of inhibiting VSV plaque formation in cultures of chick embryo cells.

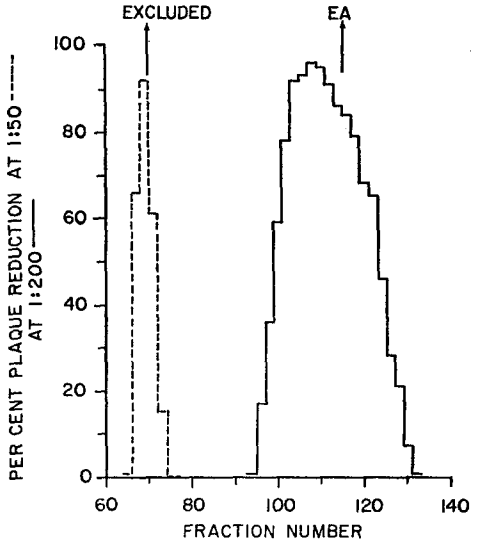

FIG. 4. Sephadex elution pattern of rabbit serum interferon. 2 ml of serum obtained 4 hr after intravenous injection of NDV were layered on the column and effluent fractions assayed for interferon activity by reduction of VSV plaque formation on RK cell cultures. Note the suggestion of a trailing shoulder in the region of peak elution of ovalbumin (EA) marked by the arrow.

These experiments indicate that nonvirus-induced interferons prepared from rabbit peritoneal macrophages are relatively labile but this property is not an absolute criterion for differentiation from virus-induced interferons which also exhibit varying degrees of heat and acid lability. It also seems clear that the interfering activity of these NDV-induced preparations is due to interferon rather than to residual viral constituents.

Sephadex Gel Filtration

NDV-Induced Rabbit Serum Interferon.—The molecular weight of circulating interferon was estimated by filtration through Sephadex G-100 of sera obtained from rabbits 4 or 6 hr after intravenous injection of NDV.

The 4-hr serum specimen was found to be free from infectious virus and had an interferon titer of 80,000 PDD_{50}/ml, which was not affected by ultracentrifugation or by potent anti-

NDV immune serum. Effluent fractions collected from the column were first tested at low dilution to locate the zones of interferon activity. Active fractions were then diluted 1:50 or 1:200 and tested for their capacity to inhibit plaque formation of VSV on four replicate cultures of RK cells.

Fig. 4 shows two distinct zones in the elution pattern of interferon in the 4-hr serum sample. A sharp peak of activity is clearly evident in fraction 69 (276 ml), the exclusion volume of this column. Since BSA dimer used as a marker protein peaked at fraction 76 (see Fig. 1), the excluded component of serum interferon can be assumed to have a molecular weight greater than 134,000. Alternatively, this component may represent a polymer of lighter interferon subunits or an aggregate of interferon and an inactive carrier protein. Most of the interferon was recovered in the second elution zone, which showed a much less sharply defined peak at fractions 107–108, corresponding to a molecular weight of 51,000–52,000. The resolving power of the column may not be sufficient to exclude the existence in serum of a third interferon species represented by the trailing shoulder in the region of peak elution of EA (mol wt $\simeq$45,000). Other minor peaks also may have been obscured by the large amount of interferon activity in the major band.

The presence of at least two distinct components was confirmed by Sephadex filtration of serum obtained 6 hr after intravenous injection of NDV; this serum sample was not subjected to ultracentrifugation or acid dialysis. Once again, a sharp peak of activity was found in the excluded volume, whereas most of the interferon penetrated the gel and was eluted in a wide band with a peak of activity equivalent to a molecular weight of 50,000–51,000.

The high and low molecular weight interferons recovered from chromatographed serum were further compared by testing the physical properties of interferon in the peak fractions. The high molecular weight interferon was not sedimented by ultracentrifugation, was not active on chick embryo cells, nor was it neutralized by anti-NDV immune serum; these properties are all indicative of the absence of interfering viral constituents in the excluded fraction. In addition, the interferon of mol wt $\geq$134,000 proved to be labile; heating at 56°C for 1 hr or dialysis against buffer at pH 2 reduced its titer from 80 to <5 PDD_{50}/ml. In contrast, identical exposure to heat or acid of the interferon component of mol wt 51,000 resulted only in a 25% reduction in titer. The lability of the high molecular weight component partially accounts for reduction in titer when unfractionated serum interferon is exposed to heat or acid.

These data are consistent with the finding of Ke et al. (9) that sera of NDV-infected rabbits contain at least two molecular species of interferon.

NDV-Induced Rabbit Kidney Interferon.—

Medium withdrawn from a culture of RK cells 24 hr after NDV infection was ultracentrifuged three times, acid dialyzed, and redialyzed to neutrality. 2 ml of this material (10,000 PDD_{50}) was layered on the Sephadex column and interferon activity was assayed in 4-ml effluent fractions diluted 1:5. Slight but definite antiviral activity could be detected in the excluded volume (fractions 68–72), but much greater amounts were present in a broad zone extending from fraction 100 to 122. Subsequent reassay revealed $<1\%$ of recovered interferon in the excluded volume. Initial assays made by serial 2-fold dilutions of fractions from this run were too inaccurate to locate the elution peak of interferon activity with any degree of confidence. Therefore, a second 2 ml sample of this interferon was filtered through Sephadex G-100; each active fraction was diluted 1:100 and assayed on three RK cell cultures.

Fig. 5 shows a Gaussian distribution of eluted RK interferon and a sharply delineated peak of activity in fraction 114 ($V_e/V_t = 0.468$), which corresponds to a molecular weight of

45,000 (cf. Fig. 2). Ovalbumin (mol wt ≃45,000) also peaked in fraction 114. Once again, a barely detectable amount of interfering activity was present in the excluded volume (mol wt ≥134,000).

This experiment was repeated with medium from an RK cell culture harvested 6 hr after NDV infection and ultracentrifuged three times but not acid dialyzed. Sephadex filtration of this preparation again revealed a sharp peak of interferon activity in combined fractions 114–115 (mol wt ≃44,000–45,000). In this run, no high molecular weight component could be detected.

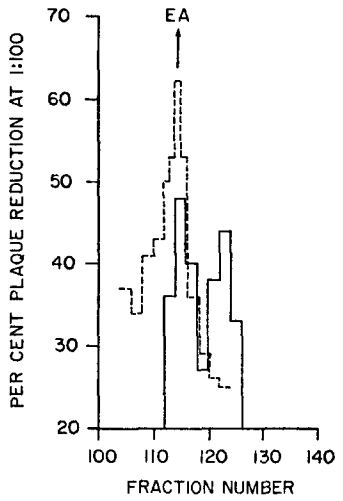

FIG. 5. Elution profiles from separate Sephadex filtration studies of NDV-induced interferons obtained from cultures of RK cells (----------) or macrophages (————). The arrow indicates the peak OD_{215} fraction of eluted ovalbumin (EA). Interferon activity of effluent fractions diluted 1:100 was tested by VSV plaque inhibition.

NDV-Induced Macrophage Interferon.—Initial Sephadex filtration studies were performed with media obtained from macrophage cultures 6 hr after NDV infection, which were then ultracentrifuged and acid dialyzed. 2 ml samples of these interferon preparations were layered on the column and effluent fractions assayed at a dilution of 1:100. In one experiment fractions 106–125 caused significant VSV plaque inhibition but, in contrast to RK interferon, no sharp peak of activity could be identified. Reduction in plaque counts of 57–63% were produced by all fractions from 116 to 121, which encompasses a molecular weight range equivalent to 38,000–43,000. This result suggested that NDV-induced macrophage interferon might consist of more than one component of similar molecular weight.

A second experiment with NDV-induced macrophage interferon again revealed a broad zone of activity when effluent fractions were assayed at low dilution. When the most active fractions were diluted to a range of activity of 60–80% plaque inhibition, two peaks of activity could be distinguished: one corresponded to mol wt ≃44,000–45,000 and the other to mol wt ≃37,000–40,000. Fractions at the excluded volume were also tested at a dilution of 1:5 and found to cause 26% plaque inhibition, again suggesting the presence of a minor interferon component of mol wt ≥134,000.

Fig. 5 shows the results of a more definitive analysis of the Sephadex filtration pattern of a third preparation of NDV-induced macrophage interferon that had been ultracentrifuged but not acid dialyzed. Effluent fractions titrated at a dilution of 1:100 revealed two distinguishable peaks of interferon activity, one in pooled fractions 114–115 (mol wt ≃44,000–45,000) and the other in pooled fractions 122–123 (mol wt ≃36,500–37,500). No interferon activity could be detected in the excluded volume of this sample. When the effluent fractions obtained from this run were reassayed at dilutions of 1:40 after storage at 4°C for 1 wk, only a single peak could be detected in fractions corresponding to a mo-

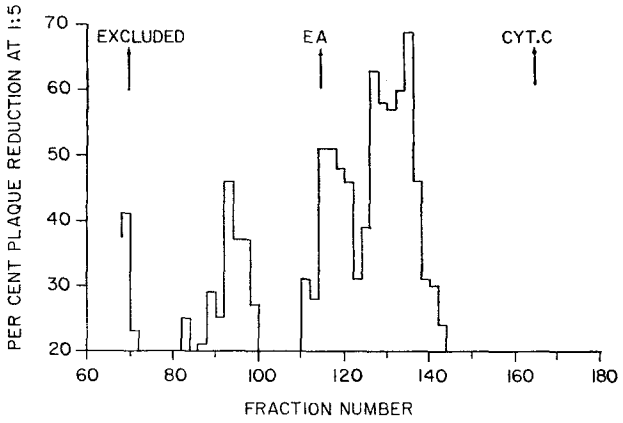

FIG. 6. Sephadex fractionation of endotoxin-induced macrophage interferon illustrating multiple peaks of plaque-reducing activity. Arrows mark the excluded fraction and peak elution of ovalbumin (EA) and cytochrome *c* (CYT. C).

lecular weight of 42,000–43,000. This finding suggests that the component of mol wt ≃37,000 is labile.

It seems clear from these studies that NDV-induced interferons produced by RK cells and macrophages share a common interferon with a molecular weight equivalent to 45,000. It also seems evident that a second, smaller, and perhaps more labile component is present in macrophage interferon.

Endotoxin-Induced Macrophage Interferon.—

Attempts to estimate the molecular weight of endotoxin-induced macrophage interferon by Sephadex gel filtration proved to be quite difficult. As previously described, this interferon is relatively labile and its titer is invariably low compared with that of virus-induced interferons. All antiviral activity in effluent fractions was lost on storage in PBS at 4°C for several days; unfractionated specimens were more stable. The most reproducible results were obtained with two preparations of interferon produced by macrophage cultures incubated for 6 hr in the presence of *E. coli* lipopolysaccharide, 100 μg/ml.

Fig. 6 demonstrates one pattern of elution from the Sephadex column of endotoxin-induced interferon which had an initial titer of 1280 PDD_{50}/ml. When effluent fractions were tested at a dilution of 1:5 on three replicate RK cell cultures, at least four zones of interferon activity could be identified. In two zones, representing the earliest effluent, peak activity was present in excluded fractions 68–69 (mol wt $\geq$134,000) and fractions 92–93 (mol wt $\simeq$72,000–73,000). Most of the antiviral activity eluted in a broad zone extending from fractions 110 to 143. Within this area three, or possibly only two, peaks could be identified in fractions corresponding to molecular weights of 42,000–45,000, 33,000–34,000, and 28,000–28,500. Comparable but somewhat variant results were obtained on repeat analysis of the same interferon sample that had been stored for 6 wk at 4°C. The second run showed four peaks of interferon activity in fractions equivalent to the following molecular weight values: >134,000, 77,000–78,000, 37,000–38,000, and 29,500–30,500.

Sephadex filtration of a low-titered (480 PDD_{50}/ml) sample of endotoxin-induced macrophage interferon revealed only two distinguishable peaks corresponding to molecular weights of >134,000 and 35,000–36,000. However, the major interferon activity was present in a broad zone extending from fractions 114 to 135 in a pattern that could well have obscured additional peaks.

The conclusion seems inescapable that endotoxin-induced macrophage interferon is polydisperse with respect to its diffusional properties in Sephadex G-100.

DISCUSSION

Several laboratories have presented evidence for molecular heterogeneity of interferons obtained from different sources. Merigan et al. (10) demonstrated conclusively that the same virus can induce the synthesis of distinguishable interferons in cultures of chick, mouse, and human cells. In addition to animal species of the producing cell, the viral or nonviral nature of the inducing agent appears to determine the physical properties of interferons. Hallum et al. (2) found that interferons in the sera of mice injected intravenously with three different inducers could be differentiated by Sephadex filtration and assigned the following molecular weight values: NDV-induced $\simeq$25,000; endotoxin-induced $\simeq$89,000; and *Brucella*-induced $\simeq$77,000 and 54,000 (two peaks). An interferon of high molecular weight has also been identified in sera of mice injected with statolon (11). Moreover, other investigators have detected two molecular species of interferon in the same serum specimen after intravenous injection of a single inducing agent such as NDV or bacterial endotoxin (9). These latter studies suggest that interferons can be synthesized at two or more tissue sites in the same animal. In fact, cell type may be the most important determinant of molecular species of each interferon. Very recent studies by Falcoff et al.[1] demonstrate most convincingly that human interferons produced by cultures of virus-infected leukocytes and amniotic cells have approximate molecular weights of 25,000 and 160,000, respectively, as determined by filtration through Sephadex G-200.

The Sephadex filtration experiments reported here seem to indicate that rabbit interferons exhibit an even greater degree of molecular heterogeneity than do

interferons produced by cells of other species. However, detection of more molecular species of rabbit interferons may be due to our use of a large Sephadex column which undoubtedly has resolving power superior to that of the smaller columns used by other investigators. Also, the reliability of our estimates of molecular weight was probably enhanced by the replicate-plate bioassay procedure (7) and by careful calibration of the Sephadex column with a wide range of marker proteins of known molecular weight (6). These factors may partially explain the discrepancy between our results and those of Ke et al. (9) who reported molecular weight estimates of $>100,000$ and 40,000 for the two major components of NDV-induced interferon in rabbit serum. In addition to the excluded fraction of mol wt $\geq 134,000$, our studies of rabbit serum interferon revealed a major elution peak in the region of mol wt $\simeq 51,000$ and a suggestive trailing shoulder in the region of mol wt $\simeq 45,000$.

The clearest and most readily interpretable patterns were obtained by Sephadex filtration of interferons induced by NDV infection of cultured cells. The excluded fractions were not detected consistently and represented only a very small proportion of these interferons. The elution profile of RK interferon can be taken as conclusive evidence that the vast bulk of this interferon is a single molecular species of mol wt $\simeq 45,000$. The Gaussian distribution of eluted biological activity lends support to the thesis that RK interferon is homogeneous with respect to size (12). A superimposable peak, equivalent to mol wt 45,000, was also found on Sephadex filtration of NDV-induced macrophage interferon. It seems likely, therefore, that NDV induces synthesis of a common interferon component in both cell types. However, NDV-infected macrophages also produced another separable interferon of mol wt $\simeq 37,000$. A plausible, but necessarily tentative, explanation of this finding might be that mobilized peritoneal macrophages are equipped with two (or more) genes, each of which can synthesize a different molecular species of interferon following NDV induction. Alternatively, the two major components may be produced by different cells in the same culture of peritoneal leukocytes.

The interferon present in rabbit serum after intravenous injection of NDV differed from NDV-induced macrophage interferons in at least two respects. As much as 10% of the serum interferon appeared in the eluate after Sephadex filtration along with proteins of mol wt $\geq 134,000$. This high molecular weight interferon was considerably more labile to heat and acid than other NDV-induced interferons. The major component of serum interferon was relatively stable and eluted in peak activity in fractions corresponding to mol wt 51,000. This property of faster diffusion through Sephadex than the major components of virus-induced macrophage interferon seems to imply that serum interferon is not synthesized primarily by macrophages. However, it is not possible to exclude the presence of a minor component of mol wt $\simeq 45,000$ which could be of macrophage origin.

No simple interpretation can be offered for the multiple and variable components of interferon produced by macrophages after induction with bacterial endotoxin. The most consistent finding was the presence of peak interferon activity in two Sephadex fractions equivalent in molecular weights to >134,000 and 33,000–38,000. These data may mean that both endotoxin and NDV can induce in macrophages the formation of an interferon of mol wt ≃37,000. By comparison, endotoxin appears to have far less capability of stimulating formation of the major constituent of mol wt ≃45,000 found invariably in medium of NDV-infected cell cultures. It is not possible at present to attach much significance to the variety of other constitutents of higher and lower molecular weight which are present in various Sephadex fractions of endotoxin-induced interferon. An obvious possibility is that they represent biologically active subunits or polymers of the primary macrophage interferon of mol wt ≃37,000. Some importance can probably be attributed, however, to the data presented in the preceding paper (1) that actinomycin can completely inhibit synthesis of endotoxin-induced as well as virus-induced macrophage interferons. This finding implies similar mechanisms of synthesis for all macrophage interferons regardless of their molecular weight.

Further refinements in techniques are required to elucidate the relationships among the various molecular types of rabbit interferons. For one, it is essential to use physical and chemical parameters other than gel filtration to study the relatedness or unrelatedness of rabbit interferons. Nevertheless, the data on gel filtration strongly suggest that the producing cells as well as the viral or nonviral nature of the inducing agent determine the type of interferon produced in a single animal species. Our experiments do not exclude the possibility that different rabbit cells contain the genetic information to synthesize an interferon of mol wt ≃45,000. Certain cells, such as macrophages, may be specially adapted to synthesize other interferons and thus serve a special function in defense against viral infection.

SUMMARY

Antiviral factors present in cultures of rabbit peritoneal macrophages or rabbit kidney (RK) cells infected with Newcastle disease virus (NDV) and those in cultures of uninfected macrophages all fulfilled the biological and physicochemical criteria for classification as interferons. Virus-induced macrophage and RK interferons were slightly more stable to heat or acid than "spontaneously produced" or endotoxin-induced macrophage interferon. Interferon activity in serum of NDV-infected rabbits was decidedly more labile than NDV-induced macrophage interferon. However, these differences in lability were too slight to serve as a useful basis for distinguishing one rabbit interferon from another.

Rabbit interferons from various sources could be differentiated by filtration

through Sephadex G-100 and their molecular weights estimated by comparison with elution profiles of a series of marker proteins of known molecular weight. Each of four different preparations of rabbit interferons was found to contain more than one molecular component. Elution peaks for three NDV-induced interferons were equivalent to the following molecular weights: RK ≃44,000–45,000 and >134,000 (variable and <1% when present); macrophage ≃37,000, 44,000–45,000, and >134,000 (variable and <1% when present); and serum ≃50,000–52,000 and >134,000 (~10% and heat labile). NDV-induced serum interferon may also contain another molecular component of mol wt ≃45,000 represented by a trailing shoulder from the major 51,000 mol wt peak.

Endotoxin-induced macrophage interferon proved to be polydisperse. Sephadex filtration of this interferon did not reveal clear and consistent elution patterns, partially owing to its low initial titer and lability. However, variable peaks of biological activity could be detected in Sephadex fractions equivalent to approximate molecular weight values of >134,000, 72,000–78,000, 33,000–38,000, 28,000–30,000, and possibly a component of 42,000–45,000. A major component of mol wt ≃37,000 was present in all samples of endotoxin-induced macrophage interfron. The other constituents may be biologically active subunits or polymers.

These data indicate that rabbit macrophages produce two primary kinds of interferon: (*a*) an RK-like component of mol wt ≃45,000 that is synthesized in greatest amount after viral induction, and (*b*) a different species of mol wt ≃ 37,000 that can also be synthesized in the absence of viral induction. The presence of major interferon constituents of mol wt ≃51,000 and > 134,000 in rabbit serum after viral induction suggests that macrophages are not the principal interferon-producing cells that respond to intravenous injection of NDV.

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