The Uptake of a Labeled Double-Stranded Polynucleotide by Cultured Rabbit Kidney Cells: An Electron Microscopic Study

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ABSTRACT Polyribocytidylate-^{3}H–polyriboinosinate (rC-^{3}H:rI) enters cultured rabbit kidney cells from the surrounding medium within ½ hr after exposure. Grains are found in the cytoplasm, nucleus, and nucleolus. At 2 hr, grains are localized predominantly over the nucleolar regions. Subsequently, the grains in the nucleus become dispersed. A specific receptor site for the initiation of interferon production was not revealed.

The intracellular fate of an interferon inducer, double-stranded polyribocytidylate-^{3}H–polyriboinosinate (rC-^{3}H:rI), has been studied by Bausek and Merigan.[1] Cellular uptake was first detected after 3 hr exposure, when the grains were localized predominantly over the nucleolus. Subsequently, both the cytoplasm and the nucleus were heavily labeled.

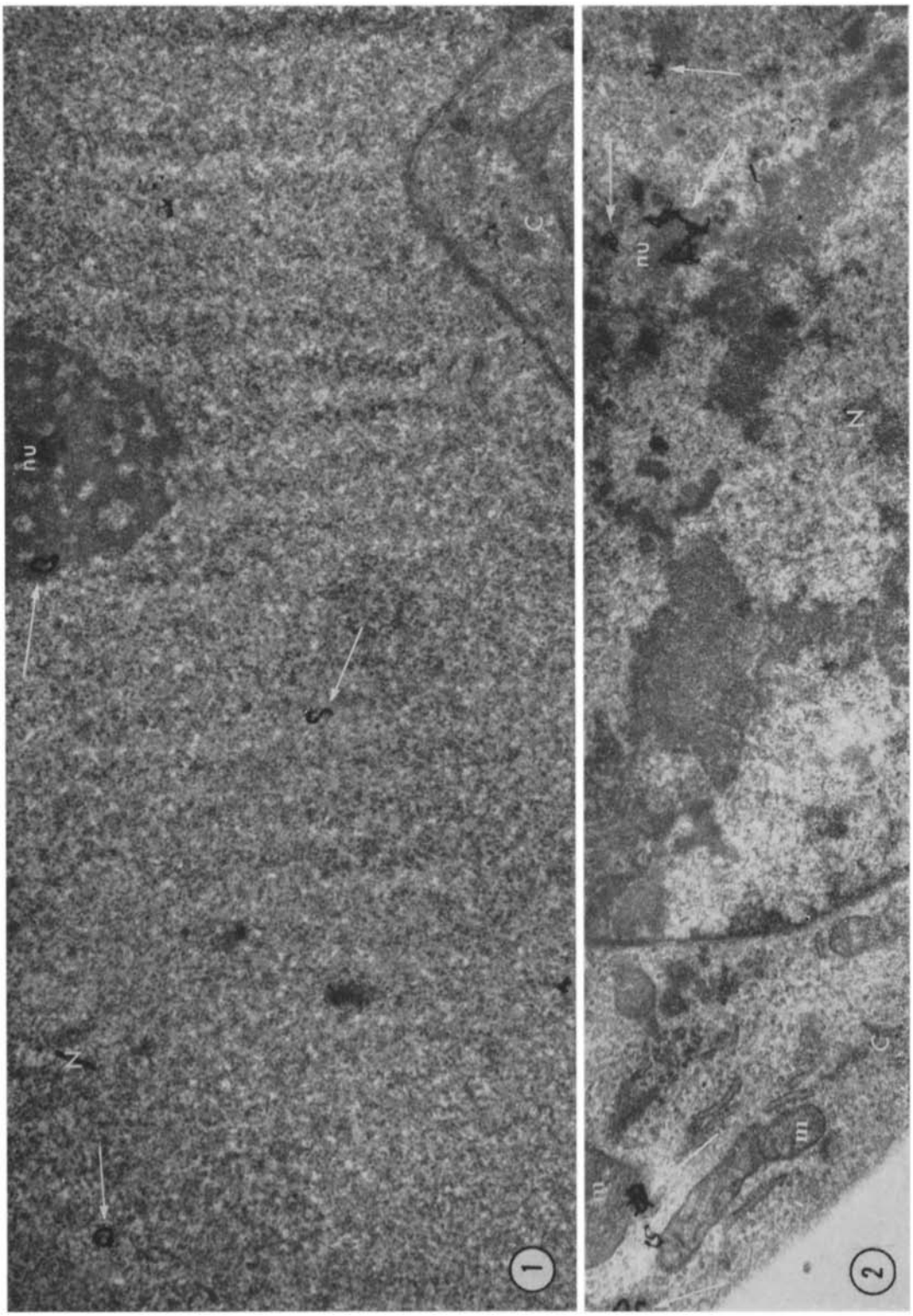
In the above light microscopic study, the initially observed uptake of polyribonucleotide was late in relation to interferon production, and a specific cellular receptor site was not revealed. In view of the several advantages of the electron microscope over the light microscope in radioautography, the study being reported herein was undertaken to determine more precisely the intracellular localization of rC-^{3}H:rI in cultured rabbit kidney cells.

METHODS

Treatment of Cell Cultures with rC-^{3}H:rI

Rabbit kidney cells were grown in 35-mm plastic plates as previously described (1). rC-^{3}H:rI (specific activity 6.97 mCi/mM P, labeled only on the pyrimidine moiety, purchased from Schwarz BioResearch, Orangeburg, N. Y.) was dissolved in phosphate buffered saline (PBS), pH 7.4, and mixed with a solution of cold rI. (To make certain that all rC-^{3}H was in a double-

[1] G. H. Bausek and T. C. Merigan. 1969. Personal communication.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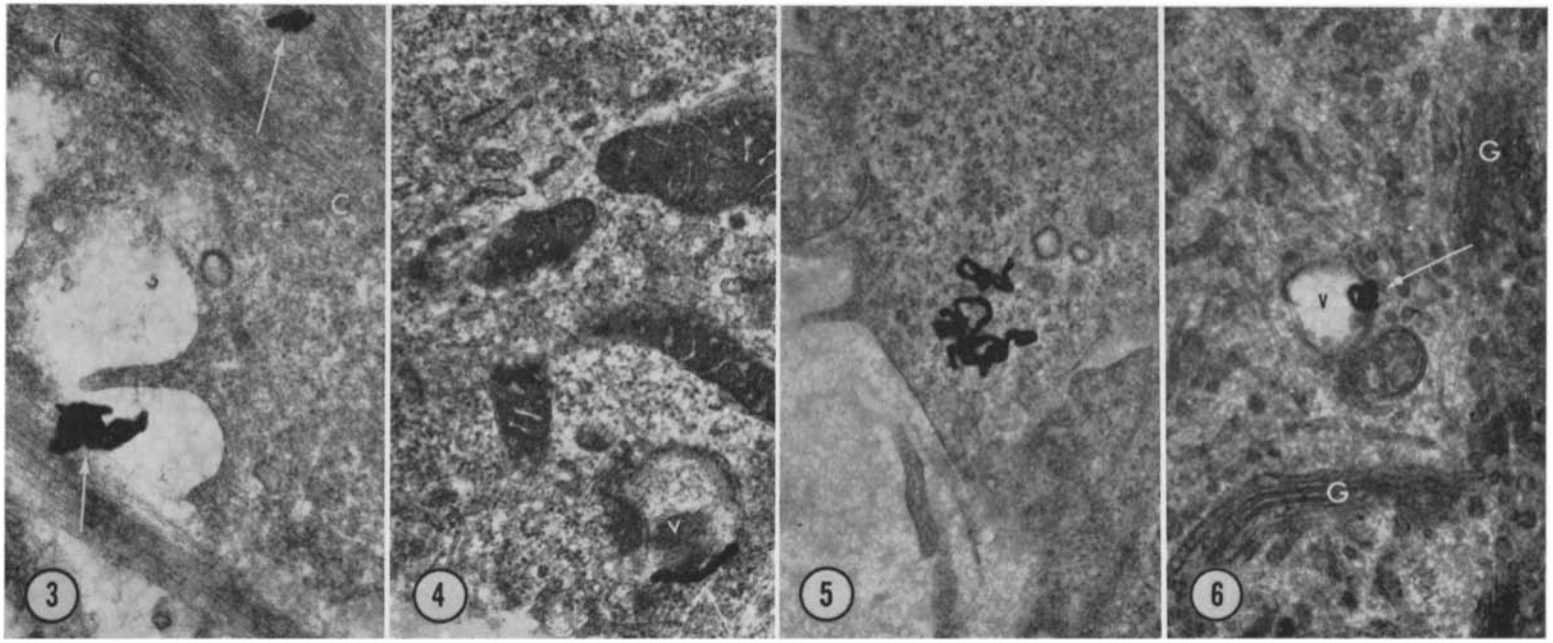

FIGURE 1. Section of part of a rabbit kidney cell exposed to rC-^{3}H:rI (3.2 μCi) for ½ hr, showing cytoplasm (*C*) and grains (arrows) over the nucleus (*N*) and nucleolus (*nu*). × 19,200.

FIGURE 2. Section of part of a rabbit kidney cell exposed to rC-^{3}H:rI (0.8 μCi) for 2 hr, showing a cluster of grains (arrows) over the nucleolus (*nu*) and fewer grains in the nucleus (*N*) and cytoplasm (*C*). Some grains are seen in the vicinity of mitochondria (*m*). × 14,500.

FIGURE 3. Section of part of a rabbit kidney cell exposed to rC-^{3}H:rI (0.8 μCi) for ½ hr showing a cluster of grains (arrows) in the extracellular space in the vicinity of cell processes. One grain is present in the cytoplasm (*C*). × 27,500.

FIGURE 4. Section of part of a rabbit kidney cell exposed to rC-^{3}H:rI (0.8 μCi) for 1 hr showing a grain (arrow) in the vicinity of an intracytoplasmic vacuole (*v*) situated near the cell surface. × 27,500.

FIGURE 5. Section of part of a rabbit kidney cell exposed to rC-^{3}H:rI (0.8 μCi) for 2 hr showing several grains within the cytoplasm in the vicinity of ribonucleoprotein particles. × 24,700.

FIGURE 6. Section of part of a rabbit kidney cell exposed to rC-^{3}H:rI (0.8 μCi) for 4 hr showing a grain (arrow) in the vicinity of a vacuole (*v*) situated close to the Golgi complex (*G*). × 27,500.

stranded form, the molar quantity of rI used was double that of rC-^{3}H.) The double-stranded rC-^{3}H:rI, but not its single stranded constituents, was found to inhibit plaque formation with vesicular stomatitis virus in rabbit kidney cells (1).

A set of cultures were treated with either 0.8 or 3.2 μCi of rC-^{3}H:rI in PBS for 1 hr at 37°C. Thereafter, the cells were washed to remove the unadsorbed polynucleotide. Further incubation was carried out in serum-free minimal essential medium (MEM). Cultures were removed for electron microscopy at different intervals, ranging from 30 min to 6 hr after the addition of rC-^{3}H:rI.

Preparation for Electron Microscope Radioautography

The cultures were washed twice in cold PBS, fixed in cold 3% glutaraldehyde in PBS for 1–12 hr, postfixed in 1% osmium tetroxide in PBS for 1 hr, passed through graded alcohols, and embedded in Epon.

Thin sections were mounted on Formvar-coated grids which had been carbon-coated prior to section application. The sections were coated with emulsion by dipping in a 1:2 dilution of Agfa-Gevaert NUC 307, stored at 4°C in a desiccator for 3–12 wk, developed in Amidol (18°C for 6 min), stained with uranyl acetate, and examined in a Siemens Elmiskop I.

RESULTS

Cellular uptake was detected as soon as ½ hr after exposure to rC-^{3}H:rI. The grains were distributed in the cytoplasm, nucleus, and nucleolus with no evidence of preferential localization (Fig. 1). At 2 hrs, labeling over the nucleolus (Fig. 2) exceeded that found over other regions of the cell. In subsequent periods, the grains were dispersed and no longer concentrated over the nucleolus.

There was some indication that the polyribonucleotide was phagocytized; grains were observed extracellularly in the vicinity of projecting cell processes (Fig. 3), within intracytoplasmic vacuoles situated close to the cell surface (Fig. 4), and within structures resembling autophagosomes. No specific cellular receptor site was found, since in addition to the structures enumerated above, labeling was observed among ribonucleoprotein particles (Fig. 5) and in the vicinity of vesicles, mitochondria (Fig. 2), and the Golgi apparatus (Fig. 6).

DISCUSSION

Within ½ hr of exposure, rC-^{3}H:rI enters the rabbit kidney cell and attains the nucleus and nucleolus. Thus, this interferon inducer is distributed within the cell shortly prior to the time when interferon production can be detected, namely, between 1 and 3 hr after exposure (1).

Since no serum was added to the culture media used in our experiments, it can be assumed that the extracellular double-stranded polynucleotide was not degraded.[1] However, one can not be certain that intracellular grains represent polynucleotide in the double-stranded state; they may represent a degradation product. Furthermore, at the 1 hr point and thereafter, intracytoplasmic grains may represent either polynucleotide which has entered the cell from the surrounding extracellular medium or labeled rC which has been incorporated into cellular RNA in the nucleus and subsequently released into the cytoplasm. In agreement with Bausek and Merigan's findings[1], the predominantly nucleolar localization of the grains in the nucleus suggests incorporation into cellular RNA.

A specific cellular receptor site for the initiation of interferon production was not revealed. Although there was suggestive evidence for the phagocytosis of rC:rI by rabbit kidney cells, a definitive statement will require further observations, since the number of labeled cells represented only a small percentage of those sectioned.

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Discussions from the Floor

Dr. Karl H. Fantes (Glaxo Research, Ltd.): I would like to ask Dr. Field whether there was any correlation between molecular size of his poly I: poly C preparations and toxicity.

Dr. Field: These studies are underway at the present. I don't really think we have anything conclusive that I could say at this point. This is obviously a very important point.

Dr. Marcel Pons (Public Health Research Institute): I'd like to ask Dr. Field if he knows whether the double-stranded RNA that one can obtain from normal chick fibroblast cells will stimulate interferon production? And secondly, if you've tried polyanionic compounds like polyvinyl sulfate to see if that will stimulate interferon production. I'm thinking of Came's recent work.

Dr. Field: In answer to your first question, when Doctors Colby and Duesberg went into this, they did find some RNase-resistant RNA in normal cells. This RNA did not hybridize with the vaccinia DNA, so this was probably not virus-induced

RNA. It was tested and was found to be inactive as an interferon inducer. Dr. Montagnier has also looked at this. I don't know anything about interferon induction here; perhaps someone else does.

Dr. De Maeyer: Since Luc Montagnier is a colleague of mine at the Radium Institute, it was the natural thing for us to look for interferon induction with the double-stranded RNA he obtains from normal rat liver cells, and which most probably is not viral RNA. The reason for saying it's not viral RNA is that it does hybridize with rat DNA to a high extent (Montagnier and Harel, unpublished results). Now, when we put this double-stranded rat liver RNA in tissue culture of chick cells, mouse cells, and rat cells as well, we find very good interferon induction and inhibition of viral replication in these cultures.

Dr. Field: You know, maybe this relates in some way to this constant low level production of interferon in cell cultures, which was mentioned previously. There was a second question that Dr. Pons had, and that was about polyvinyl sulfate. No, we have not found polyvinyl sulfates active as interferon inducers in rabbits.

Dr. Baron: Concerning the hypothesis which Dr. Field discussed, that the infection by single-stranded RNA virus may induce interferon only via the induction of double-stranded, replicative form of viral RNA, when this hypothesis was first proposed, it remained possible that the greater ribonuclease stability by the double-stranded RNA's might account for their larger inducing effect as compared with single-stranded RNA. Therefore, if single-stranded RNA's could reach the cell without being destroyed, they would also induce interferon.

It has been shown that single-stranded, RNA's, of synthetic, natural, viral origin, or cellular origin, can induce, if one has a system where RNAse activity is diminished and where the permeability of the cell is increased (Billiau et al., International Symposium Interferon, Lyon, France, 1969. In press). Also the work by Dianzani and Burke indicate that input viral RNA, presumably single-stranded, could conceivably induce under certain conditions. Since most naturally occurring, single-stranded RNA's have some secondary structure, and since many have been shown to induce interferon, by the criteria offered, we would have to relabel these RNA's as double-stranded or agree that previously defined single-stranded RNA can induce interferon. The strandedness of the nucleic acid inducers of interferon are of importance for two reasons. It reflects on the natural mechanism by which viruses induce interferon, and it helps in the search for new inducers.

Dr. Levy: The secondary structure we have referred to is the multistrandedness arising when one molecule of hydrogen bonds to a second molecule, not the base-stacking type of secondary structure. I think that when you get into viral RNA's, you have something more close to random copolymers than anything else. You can't have the sequences alternating in any regular way, because the sequences have to carry genetic information.

Dr. Field: Well, I think this argument about double- and single-strandedness has gone round and round for quite awhile without much progress. Perhaps Dr. Merigan has explained why single-stranded RNA as an inducer may be active to some extent. Our insistence has been on the activity of double-stranded molecules. Perhaps this ought to be broken down into two different areas. If one looks at the practical problem of induction, there is induction of interferon in animals and induction of

resistance against virus infections. The double-stranded, complementary RNA's are excellent inducers. The single-stranded RNA's are, in our hands, noninducers, and I presume also in Dr. Baron's hands they are noninducers of resistance against virus infection.

We come down to the academic question of whether single-stranded RNA molecules can ever induce versus, the induction that is obviously present with double-stranded molecules. I think we have to look at several points. The quantitative difference is tremendous. You have about 1000-fold increased activity in double-stranded versus single-stranded RNA. Now, if you want to look at the single-stranded molecule, you have to account for this difference and what might lie within the structure of that molecule. I think Dr. Merigan's explanations of areas of stability, strandedness, however you want to define this, may explain why these single-stranded RNA molecules may induce under certain circumstances. As for induction during virus infection, this has previously been discussed. Another question is the possible strandedness or complexity of the input viral RNA. I believe what Dr. Lockart saw with RNA from an arbovirus was a DNA-like melting profile with thermal transition midpoint at about 60°C. I do not suggest that this RNA was complementary and double-stranded in terms of two separate, covalently bonded polynucleotides, but I do suggest that there are areas of strandedness and that this may be involved in the induction of interferon.

From the floor: Dr. Field, I would like to ask one thing. Most of your studies have been done with synthetic polynucleotide inducers. What is your comment about the observation that polynucleotide-induced interferon is resistant to actinomycin D and various protein synthesis inhibitors, while viral induced interferon is sensitive to various metabolic inhibitors. This suggests that, as pointed out by Dr. Youngner, there are several, at least two, types of interferon production in cells—one that is preformed and another one that involves *de novo* synthesis.

Dr. Field: I'm not in a position to answer that. I should ask Dr. Vilček, since he just gave a paper on this.

Dr. Jan Vilček (New York University School of Medicine): I think this is a very difficult question. The fact that there is a difference in the sensitivity of viral-induced and polyribonucleotide-induced interferon production to inhibitors of RNA and protein synthesis does not necessarily prove that there is a difference in the actual process of the production of this interferon. It may reflect a difference in the processing of the inducer. I don't say that this is the case, but we cannot exclude this possibility. So I think the problem is wide open, and my personal opinion is that there has been enough circumstantial evidence accumulated in favor of the view that nucleic acid is the stimulus for interferon production in a virus infected cell, although other factors may be involved as well.

Dr. Levy: In connection with those last presentations, I'm not quite sure about the meaning of uptake by cells of radioactivity of poly I: poly C. We have looked at the uptake of radioactive double-labeled poly I: poly C made with ^{14}C in the poly I and tritium in the poly C. We looked at uptake of this molecule in a variety of different cells. Basically, cells break down poly I: poly C, and they break it down fairly rapidly. Different cells break it down to different extents; some cells break down the poly I more rapidly, some cells break down the poly C more rapidly. I'm not con-

vinced that this is going to be ultimately meaningful as far as biological activity is concerned.

Dr. De Maeyer in his presentation showed that the C-57 black mice produce a good deal more interferon than the Balb/c mice. I will present some work on the mechanism of the antitumor action of the poly I:poly C. In connection with that study, we have found that if you give mice poly I:poly C overnight and then studied protein synthesis or RNA synthesis from the various organs of those mice, you'll find that poly I:poly C exerts a strong effect. In the C-57 black mice, overnight treatment with poly I:poly C leads to marked augmentation of proline incorporation into many organs. With the Balb/c mice which are poor interferon inducers, poly I:poly C treatment overnight leads to marked inhibitions of protein synthesis in most of the organs. I'm not quite sure how this ties in, but there certainly appears to be some sort of correlation.

And just to clarify this question of embryotoxicity, Dr. Adamson is in the audience, and I hope he'll forgive me if I tell something about what he told me the other day. Dr. Adamson did report the embryotoxic effects of poly I:poly C in rabbits. Dr. Hertz, on the other hand, found that mice show no such embryotoxic effects, and Dr. Adamson now finds that rats also show no such embryotoxic effects. I think the toxicity of this compound in different species is going to be quite different. Mice are quite rugged against the action of poly I:poly C. Rabbits, I think, are quite sensitive; maybe after a little while we'll know how humans react.

Dr. Pons: This is just a suggestion. I perhaps didn't make myself clear before. Came has shown that polyvinyl sulfate is capable of stimulating interferon production. As we showed recently, polyvinyl sulfate is capable of completely replacing the RNA on the ribonucleoprotein of influenza, thereby producing a protein-polyvinyl sulfate complex. I think it would be very interesting to study the effects of a single-stranded molecule, polyvinyl sulfate, which we know can stimulate interferon to a certain extent, and couple it with the protein portion of the ribonucleoprotein, giving you, in a sense, a double-stranded molecule which has all the sedimentation properties and morphological properties of the RNP, and see what that does.

Dr. Carl A. Pinto (Smith, Kline & French Laboratories, Philadelphia, Pa.): I would like to address another question to Dr. Field. Have you done any studies with poly I:poly C in subhuman primates, and if so, what is its capacity to induce interferon in these species?

Dr. Field: We have looked at the induction of interferon in African Green monkeys and also the Rhesus monkeys. With the test systems we have available, we have not seen induction.

Dr. Gerald Mayer (William S. Merrell Co., Cincinnati, Ohio): I'd like to ask Dr. De Maeyer if an initial myxovirus stimulation will cause a refractory state to subsequent EMC or poly I:C stimulation of interferon. In other words, do you see tolerance to a subsequent injection of EMC or poly I:C if myxovirus is injected first?

Dr. De Maeyer: That's an interesting problem, to see if viruses inducing interferon production of different radiosensitivities can cause refractory states to each other's interferon induction. We haven't examined this yet, but it's on our program.

Dr. Vilček: Dr. De Maeyer, you showed that the difference in the capacity to produce high or low quantities of interferon in different lines of inbred mice is con-

trolled by a single gene. Did you compare the ability of tissues isolated from these mice in vitro to produce interferon, and was there a quantitative difference also in vitro?

Dr. De Maeyer: Yes, we tried cells of different origins in an in vitro system. First of all, peritoneal macrophage cultures, derived from Balb/c and from C57BL mice, when stimulated with Newcastle Disease virus, produced about equal amounts of interferon; in other words, the in vivo difference in interferon production was not reflected in the macrophage culture. The second system we tried was mouse embryo fibroblasts. NDV-induced interferon production was studied in cultures derived from either Balb/c or C57BL mice. Using this approach, we obtained conflicting results; sometimes C57BL fibroblasts did produce more interferon than Balb/c fibroblasts, and sometimes the interferon productions were about the same. Because the results were so irregular, we abandoned the system. The third in vitro system we tried were heparinized whole blood suspensions obtained from either C57BL or Balb/c mice, to which NDV was added and left for about 18 hr at 37°C. Using this approach, whole blood suspensions derived from C57BL mice produced on the average about three times as much interferon as did whole blood suspensions derived from Balb/c mice. This phenomenon is quite reproducible and furthermore, in backcross animals there is a correlation between high interferon production in vivo and in vitro. Unfortunately, as I indicated, the difference is only about threefold, and therefore the test is just not good enough to determine mouse genotype in vitro (De Maeyer, E., and J. De Maeyer-Guignard. 1970. *Ann. N. Y. Acad. Sci.* In press).

Dr. Vilček: But in any case this rules out the possibility that the difference among these mice would be not in interferon production but in, say, the rate of clearance of serum interferon.

Dr. De Maeyer: Also, we did study clearance of interferon in Balb/c and C57BL mice by inoculating interferon and measuring rates of disappearance, and we found no difference between the mouse strains.

Dr. Levy: This is in a nature of a semiphilosophic question to Dr. De Maeyer. We've been speaking about the amount of interferon produced. You're really measuring the biological activity of interferon produced. Have you considered the possibility that really the nucleotide sequence in the genes of the low producers is just coding for an interferon molecule that is somewhat less effective than that coded for by the nucleotide sequence in the gene of the high producer. You might be making the same number of interferon molecules, but that one just does not work as well as the other.

Dr. De Maeyer: I know, we have also thought of this; maybe the same number of interferon molecules is made by Balb/c mice, but they are less efficient in inducing the antiviral state. I cannot answer your question, and I do not know how one could, since the only way of measuring interferon is by its antiviral activity. We did compare molecular weights of NDV-induced serum interferon of Balb/c and C57BL mice, and we found in both cases a heavy and light peak. The heavy peak was proportionally less important in C57BL mice, and this may be some evidence for a qualitative difference. Another experiment we did, but that doesn't really answer the question either, was to measure C57BL and Balb/c serum interferon activity in both C57BL- and Balb/c-derived fibroblast cultures. Both interferons had comparable activity in Balb/c and C57BL cells.

Dr. Field: In Dr. Vilček's paper he mentioned that there was repression of early

interferon production in poly I:poly C-induced rabbit kidney cells, by cycloheximide. I would like to comment on some work we have done along these lines. In our experiments, primary rabbit kidney cells as monolayers were treated with 1 μg poly I: poly C/ml instead of with the 40 μg/ml which he used, incubated for 2 hr, and then washed free of residual inducer. These cells were trypsinized off the glass, put into suspension in spinner flasks, and either incubated in the presence of cycloheximide at 10 μg/ml or without the cycloheximide. What we saw there was a marked suppression of the production of interferon. We're looking at hourly production of interferon within the first 3–4 hr, and we saw a marked suppression of production of interferon. What happened after that I was surprised at, and apparently Dr. Vilček's data explain what was happening. We saw tremendous increases in interferon production.

Dr. Friedman: Just a comment. In speaking about the induction of interferon by virus infection, I think people are a little too fast in talking about a replicative form and a partly double-stranded RNA replicative intermediate. I think the data of Weismann have brought up really serious objections to the idea that these things exist as such in the cell. He has shown that during the extraction of RNA, one probably hybridizes negative and positive strands. For the most part, the negative strand seems to exist as a single-stranded molecule within the cell in its natural state. There is a very strong evidence in favor of that. One should keep this in mind when speaking about natural induction of interferon by viral double-stranded RNA. It may really not exist in this form.

Dr. George Miroff (Union College): Dr. De Maeyer, you made a considerable point about differences between the Balb/c and the C57 strains of mice. I presume then you referred to the tumor incidence in the two strains and tried to correlate that with the interferon production in these two species. Is that correct?

Dr. De Maeyer: We have not correlated it yet; this is what we are attempting now, using F2 and backcross generations.

Dr. Miroff: Well, I presume then that you meant the ability to produce the virus was different in these two particular species, is that correct?

Dr. De Maeyer: No, what I'm saying is that it is well known that most C57BL strains are quite resistant to the induction of leukemia by Friend or Rauscher virus, to polyoma virus, and to the mammary tumor virus. As you know, Balb/c mice in general are quite susceptible to those agents. And what we are doing now, using backcross and F2 generation animals, is to find out if segregation of resistant animals and high interferon producers is to some extent correlated or not.

Dr. Miroff: Well, no doubt you are aware of the fact that C-57 mice will not develop a tumor when the mammary tumor agent is introduced and will continue to produce a transmissible factor as evidenced by the production of tumors in agent-free, susceptible young which she has foster nursed. This was done years ago by Andervondt. In addition, Haagensen and Moore used C-57 as a bioassay animal for tumor production.

Now, I think the two questions, tumor production and the production of the transmissible factor, should be clearly distinguished. As far as the production of virus or transmissible material is concerned, C-57 and the Balb/c are really not too different.

Dr. De Maeyer: As far as what is concerned?

Dr. Miroff: The ability to transmit or produce the virus or transmissible factor for production of mammary tumors.

Dr. De Maeyer: I believe it depends on what C-57 strain you are talking about. You are talking about the C-57 strain which is susceptible to mammary tumor agent, which is Dr. Moore's strain I believe.

Dr. Miroff: But we have C-57 mice that do not develop tumors but that will continue to produce mammary tumor agent.

Dr. De Maeyer: But the C-57/BL strains we are working with are resistant to induction of mammary tumors.

Dr. Miroff: Our C-57 mice are too, but do your C-57 mice continue to produce the transmissible factor?

Dr. De Maeyer: I don't know.