Characterization of Two Distinct Molecular Populations of Type I Mouse Interferons

By W. E. STEWART II,* SYLVIA LEGOFF and MARZENNA WIRANOWSKA-STEWART*

Institute de Recherches Scientifiques sur le Cancer, Villejuif, France

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SUMMARY

The molecular heterogeneity of acid-stable (Type I) mouse interferons induced in C2a cells by Newcastle disease virus was analysed by SDS-polyacrylamide gel electrophoresis under non-reducing and reducing conditions, and the profiles of antiviral activities obtained were characterized biologically in mouse cells and in heterologous (guinea-pig) cells. Two bands of activity, A and B, were consistently present in all interferon preparations tested: under reducing conditions, the activity in all fractions of band A (with a peak of activity at about 38000 daltons) was uniformly increased, while that of band B (with a peak at about 22000 daltons) was uniformly diminished. All the active fraction in band A had only slight activity (less than 10% of homologous titres) on guinea-pig cells, whereas all those in band B were significantly more active on guinea-pig cells than on homologous L cells. Thus, mouse type I interferon preparations contain two molecular populations of interferons that can be distinguished physically (by size), biochemically (by the effect of reduction on reactivation from SDS) and biologically (by activity in heterologous cells).

INTRODUCTION

Several laboratories have recently demonstrated that mouse cells produce distinct molecular species of acid-stable (Type I) interferons. These interferons have been shown to be distinguishable in size (Stewart II, 1974; Knight, 1975), in effect of exposure to anionic detergents in the presence of reducing agents (Stewart II, 1974; Stewart II, De Somer & De Clercq, 1974) and in binding affinity to various ligands (Davey, Sulkowski & Carter, 1976). Additionally, Type II mouse interferon has been described which differs from Type I mouse interferons in that it is destroyed at acid pH (Salvin, Youngner & Lederer, 1973; Youngner & Salvin, 1973), and it is reportedly inactive on guinea-pig cells, whereas Type I mouse interferon was active on these heterologous cells (Salvin et al. 1975).

The extent of heterogeneity of Type I mouse interferons has not been resolved. It was first claimed that there were two distinct molecular species of interferon in mouse L cell interferon preparations (Stewart II, 1974). However, the breadth of the two peaks of interferon activities isolated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) suggested that each peak might itself be composed of a number of interferons, and it has been claimed that at least 10 interferon glycoproteins can be distinguished within these two peaks by their relative migrations in SDS-PAGE (Knight, 1975). More recently it was

* Present address: International Laboratories for the Molecular Biology of Interferon Systems (ILMBIS), Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, N.Y. 10021, U.S.A.
claimed that mouse Type I interferon preparations could be separated into two components on the basis of their relative affinities for immobilized ligands (Davey et al. 1976).

As physical, biochemical and biological markers (i.e., size, stability in detergent, and cross-species activity, respectively) have thus been described which can distinguish different forms of mouse interferons, we have undertaken to apply these to resolve the extent of heterogeneity of mouse Type I interferons. Our data demonstrate that there are two populations of Type I mouse interferons which are distinguishable by physical, biochemical and biological markers.

METHODS

Cells and viruses. Mouse L929 cells and Lr-10 cells, originally derived from L929 cells, were obtained from Dr G. Bodo, Vienna. Primary guinea-pig embryo cultures were prepared from whole embryos and used through 10 passages. All cells were grown in Eagle's minimal essential medium (MEM) containing 10% calf serum. Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) stocks were prepared as previously described (Tovey, Begon-Lours & Gresser, 1974).

Interferon production and assays. Four batches of mouse C243 cell interferons, induced by NDV, using a 'superinduction' procedure and purified as previously reported (Tovey et al. 1974) were obtained through the generosity of Dr M. Tovey (Villejuif). These batches, designated RB 2, RB 19, T-64 and T-71, contained 10^8, 10^6, 10^6 and 10^6 units of interferon/ml and 10^60, 10^65, 10^70 and 10^70 units/mg of protein, respectively. For some experiments, crude, unpurified C243 cell interferon was prepared according to the method of Tovey et al. (1974); they contained about 10^4 units/mg of protein.

Interferons were assayed on either L929 cells, Lr-10 cells or guinea-pig embryo cells challenged with VSV, by modifications of the microtitration assay procedure originally described by Armstrong (1971). One unit of interferon as quoted in the text equals approx. 4 reference standard mouse interferon units.

SDS-PAGE. Interferon preparations were electrophoresed as previously described (Stewart II et al. 1976). Samples in 0.01 M-phosphate buffer, pH 7.2, were constituted to contain 1% SDS and 5 M-urea (non-reducing conditions) or 1% SDS, 5 M-urea and 1% 2-mercaptoethanol (reducing conditions) and were boiled for 1 min. A sample of 0.1 ml was applied to the gel and following electrophoresis, fractions (5 mm) were eluted in 2 ml MEM containing 10% calf serum. Conditions for electrophoresis were as described previously (Stewart II, 1974). Marker proteins (bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, and cytochrome c) were electrophoresed in parallel gels in each experiment under both non-reducing and reducing conditions.

RESULTS

The variable molecular composition of mouse Type I interferons

Mouse L929 cell interferon preparations induced by NDV (Stewart II, 1974), mouse Lpa cell interferon preparations induced by MM virus (Knight, 1975) and mouse C243 cell interferon preparations 'superinduced' by NDV (Stewart II et al. 1976) all have similar heterogeneity of activity profiles on SDS-PAGE. These profiles are qualitatively similar whether assayed on L929 cells (Stewart II, 1974), secondary mouse embryo cells (Stewart II, 1974), Lpa cells (Knight, 1975), Lr-10 cells or L1210 cells (Stewart II et al. 1976).

During several months of determining the extent of the molecular heterogeneity of different batches of C243 cell interferons, we found that the relative amounts of interferon
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Fig. 1. Profiles of antiviral activity recovered from SDS-PAGE of mouse Type I interferon preparations. Four preparations which had been induced in C3H cells by Newcastle disease virus were simultaneously electrophoresed in parallel gels under non-reducing conditions (—) and reducing conditions (— —) as described in Methods. The total units applied to gels were: RB19, 10^5.2 units; T-64, 10^5.2; RB 2, 10^5.5; T-71, 10^5.3. The total calculated recoveries from the unreduced gels were: RB 19, 15%; T-64, 20%; RB 2, 25%; T-71, 30%; and from the reduced gels: RB 19, 55%; T-64, 50%; RB 2, 50%; T-71, 90%.

Activities in different areas of the electrophoretic profile varied significantly. Activity was consistently found in two bands, and band A (with a peak of activity at about 38,000 daltons) consistently contained more activity than band B (with a peak of activity at about 22,000 daltons), but peak B composed from as much as about 40% of the total activity recovered from gels to only trace amounts (< 1%) of the recovered activity.

These variations seemed possibly due to: (i) different amounts of the components in
Table 1. Reactivation of the homologous and heterologous antiviral activities of mouse Type I interferon preparations after dissociation by 1% SDS and 5 M-urea under non-reducing or reducing conditions

<table>
<thead>
<tr>
<th>Interferon preparation:</th>
<th>Antiviral activity (log units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sp. act. (units/mg protein)</td>
<td>Dissociation conditions*</td>
</tr>
<tr>
<td>10^4</td>
<td>None (control)</td>
</tr>
<tr>
<td></td>
<td>Non-reducing</td>
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<tr>
<td></td>
<td>Reducing</td>
</tr>
<tr>
<td>10^6</td>
<td>None (control)</td>
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<tr>
<td></td>
<td>Non-reducing dissociation</td>
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<tr>
<td></td>
<td>Reducing dissociation</td>
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</tbody>
</table>

* Mouse C43 cell interferon preparations were dissociated by immersion for 1 min in a boiling water-bath under non-reducing conditions or with added 1% mercaptoethanol (reducing conditions). Samples were then diluted in medium containing 10% calf serum and assayed on L929 (homologous) or guinea-pig embryo (heterologous) cells.

different interferon batches as a result of different stabilities of the components during storage or as a result of selective purifications; or, (ii) variations in experimental conditions during SDS-PAGE causing varying efficiencies of recoveries of the components; or, (iii) differences in production of the components by the induced cells as a result of subtle differences in the production procedures.

To resolve these possibilities, we again prepared samples for electrophoresis from four batches of interferons that had previously been analysed individually, and analysed these simultaneously by SDS-PAGE. As shown in Fig. 1, the electrophoretic profile for each interferon preparation contained two bands of activity (band A with a peak of about 38,000 daltons, and band B with a peak at about 22,000 daltons), but the relative amounts of activities in band A and band B were different for each batch. The activity in band A was always about twice as widely dispersed as that in band B, even when the height of the two peaks was the same (e.g. sample RB 2 unreduced). It is significant that each activity profile obtained during the simultaneous electrophoresis of the samples on parallel gels was identical with that obtained for the same interferon preparation when electrophoresed individually several weeks previously. Therefore, the different components in mouse Type I interferon preparations can comprise varying percentages of the total interferon activity, and each component is stable for several weeks at 4 °C. Similar variations have also been seen in the electrophoretic profiles of activity of freshly prepared crude C243 cell interferon preparations, indicating that one component is not favoured by the purification procedures employed. Together, these data suggest that subtle differences in the procedure for producing interferon may allow for enrichment of particular interferon populations.

Evidence for two Type I interferon populations: effects of reduction

The distribution of the interferon activities in two bands with distinct shapes suggests that there are basically 2 distinct populations in the preparations, each with various degrees of microheterogeneity, perhaps due to differing contents of carbohydrate. It has been reported that mouse L cell interferons contain two interferon forms which differ in the effects of reduction on their abilities to regain activity after treatment with SDS (Stewart II, 1974; Stewart II et al. 1974). We therefore analysed the effects of 2-mercaptoethanol on the
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Fig. 2. Profiles of homologous and heterologous cell antiviral activity recovered from SDS-PAGE of mouse Type I interferon preparation under (a) non-reducing conditions or (b) reducing conditions. Gel eluates were assayed for antiviral activity on L929 mouse cells (---) or on guinea-pig embryo cells (---).

Evidence for two Type I interferon populations: differences in heterospecific activities

Both the distribution of the activities and the symmetry of the two effects of reducing conditions on the activities suggested that the interferon preparations contained two distinct interferon species differing both physically (in sizes) and biochemically (in effects of reduction). We therefore examined whether these interferons could be distinguished biologically, in terms of their heterospecific activities. It has been reported that the two molecular populations of human leukocyte interferons have different relative activities in both rabbit (Stewart II & Desmyter, 1975) and cat (Desmyter & Stewart II, 1976) cells; also, Type I mouse interferons can be distinguished from Type II mouse interferons by their activities on guinea-pig cells (Salvin et al. 1975). We therefore employed assays on guinea-pig cells to determine whether the Type I mouse interferon preparations might contain populations which are also distinguishable in this respect.

We found that the original mouse interferon preparations, both crude and purified,
exerted about 20 to 33% of their homologous activities on guinea-pig cells (Table I). However, whereas homologous activity was more efficiently recovered from SDS under reducing conditions than from non-reducing conditions, activity on guinea-pig cells was more efficiently restored under non-reducing conditions. These findings suggested that the predominant form of interferon inducing activity on mouse cells, i.e. band A, which increases activity under reducing conditions (see Fig. 1), might be the less important component inducing activity in the heterologous cells.

In fact, when gel eluates were assayed in parallel on mouse and guinea-pig cells (Fig. 2), it was found that each fraction in band A exerted only a small amount (≤ 10%) of its homologous (mouse) cell activity on the guinea-pig cells, whereas all the fractions in band B were significantly more active (300 to 1000%) on the heterologous cells than on the homologous cells. Additionally, each fraction in band A increased in heterologous activity on reduction and each fraction in band B lost heterologous cell activity on reduction (Fig. 2b). The only fractions showing neither the relatively low (≤ 10%) nor the high (≥ 300%) activity in heterologous cells were those in the overlap region between the two peaks of activity. Again it is noteworthy that even when the activity peak of band B was significantly higher than that of band A (Fig. 2a, assays on guinea-pig cells), band A was significantly wider than band B.

DISCUSSION

The molecular heterogeneity of both mouse and human Type I (acid-stable) interferon has recently been demonstrated using conditions that ensure dissociation of non-covalently associated molecules, i.e., electrophoresis in SDS-polyacrylamide gel (Stewart II, 1974; Knight, 1975, 1976; Stewart II & Desmyter, 1975; Reynolds & Pitha, 1975; Desmyter & Stewart II, 1976; Vilcek, Havell & Yamazaki, 1977). These heterogeneities are reflected in size, stability, and cross-species activity. Additionally, both mouse (Salvin et al. 1973, 1975; Youngner & Salvin, 1973) and human (Epstein, Stevens & Merigan, 1972; Valle et al. 1975) cells have been shown to produce acid-labile (Type II) interferons; whether or not these are heterogeneous is not yet known. The demonstration that such markers as biological assays can be used to distinguish different populations of interferons will facilitate their identification. For example, it has recently been demonstrated that two components can be separated from mouse Type I interferon preparations by chromatography on bovine serum albumin-agarose, and it has been proposed that these correspond to those revealed by SDS-PAGE (Davey et al. 1976). By determining the relative abilities of these two components to act on guinea-pig cells, further characterization could be established, and such studies are presently being made in our laboratories.

The nature of the heterogeneity is not established. However, owing to the marked dissimilarity between the two bands of activities in terms of size, stability, and heterospecific activity, and in view of the internal similarities within all areas of each band, it is tempting to speculate that the two bands represent two different primary structures (i.e., polypeptide sequences), each of which is embellished to various extents with carbohydrate moieties. Such differences in carbohydrate content would account for the width of the bands of activity, and for the finding of Knight (1975) that each of 10 to 11 areas in these bands re-migrated to its original position after isolation from SDS-gels. If carbohydrate is indeed responsible for microheterogeneity within the two interferon populations, it might be possible to narrow the bands of activity by removing this chemically or enzymically and we are attempting to do this.

As all areas within each individual band behave identically in terms of heterospecific
activity and as various degrees of carbohydrate addition seem likely in view of different intensities of staining with periodic acid–Schiff (Knight, 1975), it would appear that carbohydrate moieties do not significantly affect the cell specificities of the interferons. It also seems that the carbohydrate components of interferons are not involved in their antigenicities, as Bose et al. (1976) have reported that a significant percentage reduction in the mol. wt. of human interferon as the result of treatment with glycosidases did not affect their affinities for immobilized anti-interferon antibodies. It will be interesting to compare the SDS-PAGE profiles of such de-glycosylated interferons and normal interferons.

The nature of the differences between the band A interferons, which we proposed to designate mouse IF-I-A (for mouse interferon, Type I, band A) and mouse IF-I-B would depend on something more radical than mere differences in carbohydrate content: if that were the case, virtually all the mol. wt. difference would have to be due to carbohydrate making mouse IF-I-A (38,000 daltons) nearly 50 % carbohydrate and IF-I-B (22,000 daltons) carbohydrate-free. We propose therefore, that mouse IF-IA and mouse IF-I-B have different primary structures. This could result from their being either products of different mouse interferon genes or from IF-I-A being an active precursor molecule which is proteolytically cleaved to IF-I-B. Attempts are currently in progress to convert IF-I-A to IF-I-B by limited enzymic or chemical digestion.

One can only speculate on the physiological significance of the different interferons. It could be that the different interferon molecules differ in their abilities to exert the various non-antiviral activities that have been attributed to interferon. However, both the different molecular forms of human leukocyte interferons and also mouse IF-I-A and mouse IF-I-B were shown to have identical ratios of cell multiplication inhibitory activity per antiviral unit (Stewart II et al. 1976), and ‘priming’ and ‘double-stranded RNA toxicity enhancing’ ratios per antiviral unit are also constant (W. E. Stewart II, unpublished data). It is also possible that the different forms of interferons may have different physiological effects due to differences in binding affinities for various types of tissues in the organism, and we are currently looking at this with the different molecular species of mouse interferon.

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