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Interferon system in patients with systemic juvenile chronic arthritis: *in vivo* and *in vitro* studies

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SUMMARY

Eighteen patients with active systemic juvenile chronic arthritis (JCA) were studied for periods of up to 1 year to see whether any relationships existed between the interferon (IFN) response, the course of the underlying disease and intercurrent infections. The control group consisted of 23 children who were each seen on one occasion. IFN-α production by mononuclear cells (MNC) cultured *in vitro* was stimulated with Newcastle disease virus (NDV). Comparison of the mean IFN-α responses from all patients with control responses showed that MNC from the JCA group produced significantly more IFN-α. Furthermore, MNC obtained from JCA patients at times of systemic flare produced significantly higher titres of IFN-α than at times when the patients were clinically stable. IFN-α production by MNC from individual patients fluctuated considerably from occasion to occasion whereas IFN-γ production by MNC induced with phytohaemagglutinin (PHA) remained more stable. There was no significant difference between patients and controls with respect to IFN-γ responses and no relationship with clinical condition. Serum IFN was not detected either by sensitive bioassays for IFN-α and IFN-γ or by an immunoradiometric assay for IFN-α.

Keywords interferon juvenile arthritis flares infections

INTRODUCTION

Interferon (IFN) exerts a wide variety of biological effects in addition to anti-viral immunity (reviewed by Stewart, 1979) and there is increasing evidence that IFN can modulate the immune response. Three antigenically distinct types of IFN have been described. Virus infected leucocytes produce IFN-α and fibroblasts produce IFN-β in response to virus. IFN-γ is produced by lymphocytes when stimulated by mitogen or specific antigen.

Systemic juvenile chronic arthritis (JCA; Ansell, 1978) is a febrile, multi-system disease of childhood. Since clinical observations had suggested that intercurrent infections preceded exacerbations of disease, we investigated this possibility. The results from this study confirm the clinical impression (de Vere-Tyndall *et al.*, 1983).

The objective of the present study was to investigate some aspects of the IFN system in patients with systemic JCA and to see whether IFN responses were related to their clinical state. Several reports have suggested that patients with a variety of autoimmune conditions have elevated levels of serum IFN (Hooks *et al.*, 1979, 1982; Ohno *et al.*, 1982; Preble *et al.*, 1982; Ytterberg & Schnitzer,

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1982) which raises the possibility that IFN has a role in the pathogenesis of these diseases. Our investigation was prompted by the observation that infections often occur before disease exacerbation in patients with systemic JCA and reports of serum IFN in patients with autoimmune disease.

MATERIALS AND METHODS

Patients and controls. Eighteen children aged between 1 and 15 years (mean 9 years) with JCA of systemic onset as defined by the EULAR criteria (1977) were bled on a total of 115 occasions (mean 6·4 occasions per patient; range 1–13 occasions). All patients were taking non-steroidal anti-inflammatory drugs; nine were also receiving prednisolone and eight receiving long acting anti-rheumatic agents (gold or D-penicillamine). The control group consisted of 23 children aged between 5 and 16 years (mean 11 years) with non-inflammatory articular disease who had not had any clinical infection during the month before venepuncture.

Virus. NDV was grown in 9 day old embryonated eggs and had a haemagglutination titre of 500 units. Semliki Forest virus (SFV) was grown in BHK 21 cells (Flow Laboratories) and the stock contained 10^{10} pfu/ml on BHK cells.

Mononuclear cell separation. Four millilitres of peripheral blood with heparin (25 u/ml; preservative free; Paines & Byrne) was mixed with 4 ml of RPMI 1640 (bicarbonate buffer; Flow Laboratories) containing glutamine (2mM), penicillin (100 u/ml) and streptomycin (100 μg/ml) and mononuclear cells (MNC) were separated on a Ficoll gradient (Ficoll-Paque; Pharmacia) by centrifugation at 400 ***g*** for 20 min. The cells were washed twice in RPMI medium and suspended at 1×10^6 MNC/ml in RPMI 1640 supplemented with 10% fetal calf serum.

Production of IFN in vitro. MNC were cultured in sterile, screw capped neutral glass vials ($1 \times 3{\cdot}5$ cm) at 1×10^6/ml, 0·2 ml per vial and duplicate cultures received either 0·2 ml of NDV for induction of IFN-α or 50 μl of PHA to give final concentrations of 0·8, 8 and 20 μg/ml for induction of IFN-γ. Phosphate-buffered saline (PBS) was added to control cultures. Cultures were incubated at 37°C in a humidified incubator maintained with CO_2 5% and air 95%. Supernatants from NDV stimulated cultures were harvested 24 h after infection, stored at −70°C, and prior to IFN assay they were dialysed for 7 days at 4°C against Sörensen's glycine buffer (0·1 M glycine in 0·1 M NaCl) pH 2 and then for 24 h against PBS. Supernatants from PHA stimulated cultures were harvested after 7 days and stored at −70°C.

Interferon assay. IFN-α was assayed on EBTr cells (Flow Laboratories) and IFN-γ on HEp 2 cells (Flow Laboratories) by the inhibition of SFV RNA synthesis (McWilliams *et al.*, 1971; Koblet, Kohler & Wyler, 1972; Suzuki, Akaboshi & Kobayashi, 1974). Confluent cell monolayers in neutral glass vials ($1 \times 3{\cdot}5$ cm) were treated overnight in duplicate with serial dilutions of test samples and IFN standards in maintenance medium (MM; Eagles minimal essential medium + 2% fetal calf serum), 0·2 ml per vial. Cells were challenged with SFV at a multiplicity of infection of approximately 10 pfu/cell in MM containing actinomycin-D (Sigma) 5 μg/ml, 0·25 ml per vial. After 3 h 0·25 ml of MM with actinomycin-D containing 2 μCi/ml [5,6-^{3}H]-uridine (Amersham International; 40 Ci/mmol) was added to each culture and incubation continued for 3 h. Monolayers were washed three times with 1 ml ice cold 5% trichloroacetic acid, once with ice cold absolute alcohol, air dried, and solubilized with 0·1 ml 1 M NaOH followed by 1·9 ml NE 260 scintillant (Nuclear Enterprises). Samples were mixed and radioactivity was measured in a scintillation counter. Replicate cell control and virus control monolayers were included in each assay and by reference to ^{3}H-uridine incorporation in these cultures the amount of IFN standard inhibiting 50% of SFV RNA synthesis was calculated. IFN-α was titrated against the human leucocyte IFN standard MRC 69/19 and titres were expressed in terms of international units (iu) per ml. The sensitivity of EBTr cells to IFN-α was 1 iu/ml. IFN-γ was titrated against a laboratory IFN-γ standard consisting of supernatant from a flask of MNC cultured for 4 days in RPMI + 10% fetal calf serum with PHA (5 μg/ml). This supernatant contained 10^3 laboratory units (u) of IFN-γ per ml. HEp 2 cells detected IFN-γ at approximately 5 u/ml and IFN-α at about 6 iu/ml.

Serum from patients and controls was stored at −70°C and screened for IFN on both EBTr and

HEp 2 cells. Routinely, two-fold dilutions were prepared from a starting dilution of 1/10. Sera (undiluted) were also screened for IFN-α by an immunoradiometric assay (Celltech).

Anti-IFN treatment of serum. Dilutions of antisera against human (Hu) IFN-α (sheep), Hu IFN-β (goat) and Hu IFN-γ (rabbit) provided by Dr M. de Ley (Rega Instituut, Leuven) sufficient to neutralise 100 units of the respective types of IFN were incubated for 1 h at 20°C with equal volumes of serum from a healthy adult donor.

Statistical analysis. The arithmetic mean titre and standard deviation of $\log_{10}$ – transformed IFN titres were calculated for each group of results. If MNC from an individual failed to respond to an IFN inducer the value of the minimum detectable IFN titre in the appropriate assay was substituted in the calculation. The analysis of variance was used to evaluate the effect of drug therapy on IFN responses and to compare the mean IFN responses of MNC from patients with control responses. The influence of the patients' clinical condition on IFN responses was assessed by the paired *t*-test.

RESULTS

IFN-α production

The mean IFN-α titres produced by MNC from each patient during the study were calculated and compared with the IFN-α responses of MNC from controls. MNC from JCA patients produced significantly more IFN-α than MNC from controls (Fig. 1a). Cultures from four of 21 control donors produced no detectable IFN-α compared with three cultures out of 108 from the 18 JCA patients. No unstimulated cultures produced IFN-α.

Individual patients' results were divided into four groups according to clinical condition when blood was obtained: (1) clinically stable; (2) clinical infection, current or one resolving within the

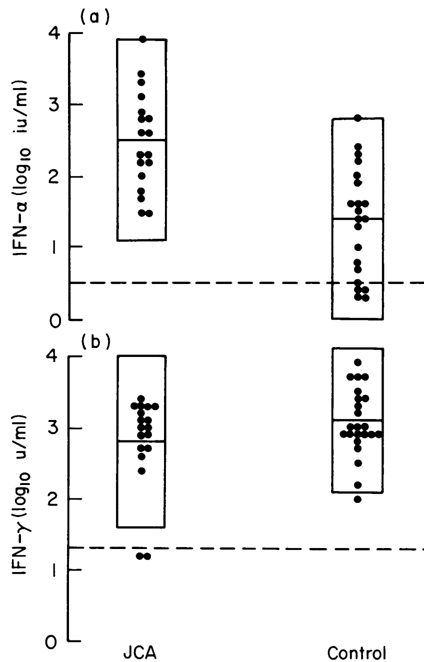

Fig. 1. IFN responses of peripheral blood MNC from patients with systemic JCA and control donors. Boxes represent mean responses ±2 s.d. The minimum levels of IFN which could be detected in culture supernatants in the IFN assays are represented by broken lines. Control donor results were obtained from single *in vitro* stimulations; JCA patient results were the mean IFN responses of MNC from each patient measured during the period of study. (a) IFN-α response to NDV; (b) IFN-γ response to PHA.

Table 1. Influence of clinical condition of systemic JCA patients on IFN-α production *in vitro* by peripheral blood MNC

Clinical state	Number of samples tested	Number of patients	IFN-α titre: Mean ± s.d. $\log_{10}$ iu/ml	IFN-α titre: Geometric mean iu/ml
Stable*	72	16	2·3 ± 0·4	200
Clinical infection†	12	9	2·8 ± 0·8	631
Disease flare—joints‡	12	6	2·5 ± 0·6	316
—systemic§	12	8	3·2 ± 1·0	1,585

* IFN-α responses of patients when clinically stable.

† Clinical infection (symptoms or objective signs present for ⩾24 h at time of study or within previous 7 days.

‡ Patients with disease flare at time of study with exacerbation confined to the joints.

§ Patients with systemic flare at time of study (typical evening fevers and increased joint activity for ⩾3 days).

previous week; (3) disease flare affecting joints only; (4) systemic flare (Table 1). The titres of IFN-α produced at times of systemic flare were significantly higher than those produced at times when the same patients were clinically stable (paired *t*-test; $P = 0{\cdot}01$). Six patients were involved in this comparison; the mean difference between the two groups was 0·97 $\log_{10}$ iu/ml and the standard deviation was 0·59 $\log_{10}$ iu/ml. No other comparisons with the clinically stable group were significant ($P > 0{\cdot}05$).

The sequential IFN-α responses of MNC from patient G.H. are shown in Fig. 2a. IFN-α production fluctuated between 16 and 3,200 iu/ml but no obvious relationship between clinical state and the IFN-α response was seen.

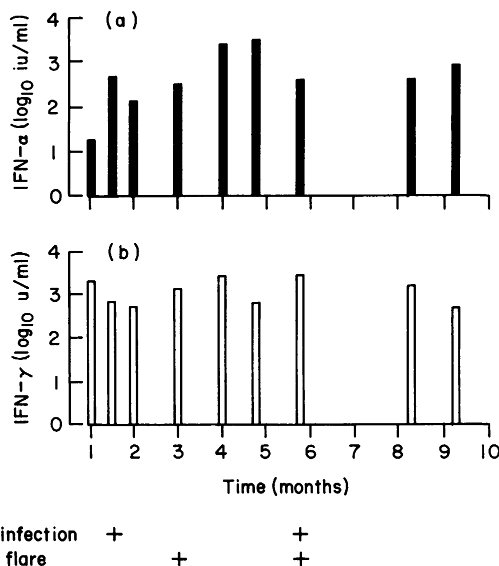

Fig. 2. Sequential IFN production by MNC from patient G. H. (♂; aged 13 years). Responses to inducers of IFN were measured on nine occasions over a period of 9 months. Clinical infections and disease flares occurred at the times marked +. (a) IFN-α response to NDV; (b) IFN-γ response to PHA.

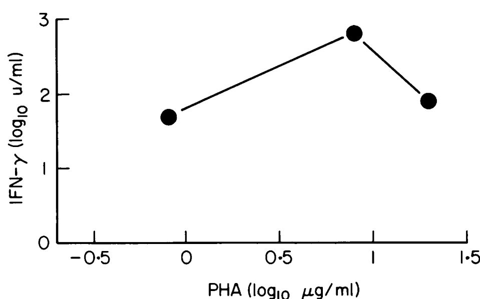

Fig. 3. Effect of concentration of PHA on the production of IFN-γ by peripheral blood MNC. The initial cell concentration was 1×10^6/ml and supernatants were harvested after 7 days of culture.

IFN-γ production

The IFN-γ responses of MNC were influenced by the concentration of PHA (Fig. 3) and so maximum responses were analysed. Unstimulated cultures from patients and controls occasionally produced low levels of IFN-γ. There was no significant difference in IFN-γ production between patient and control groups ($P > 0{\cdot}05$; Fig. 1b). Cultures from all control donors produced IFN-γ in response to PHA whereas on five out of 114 occasions MNC from JCA patients failed to produce IFN-γ irrespective of the PHA concentration used. The youngest patient, J.Y. aged 1 year, was seen on three occasions and MNC from this patient consistently failed to generate detectable levels of IFN-γ

The maximum IFN-γ response of PHA stimulated MNC was not related to the clinical state of the patients (paired *t*-test; $P > 0{\cdot}05$). This lack of correlation was also seen when IFN-γ responses obtained with each PHA dose were compared (results not shown).

In contrast to IFN-α less variation of the IFN-γ response was seen during longitudinal studies of JCA patients, e.g. maximum titres of IFN-γ produced by MNC from patient G.H. ranged between 500 and 2,500 u/ml (Fig. 2b).

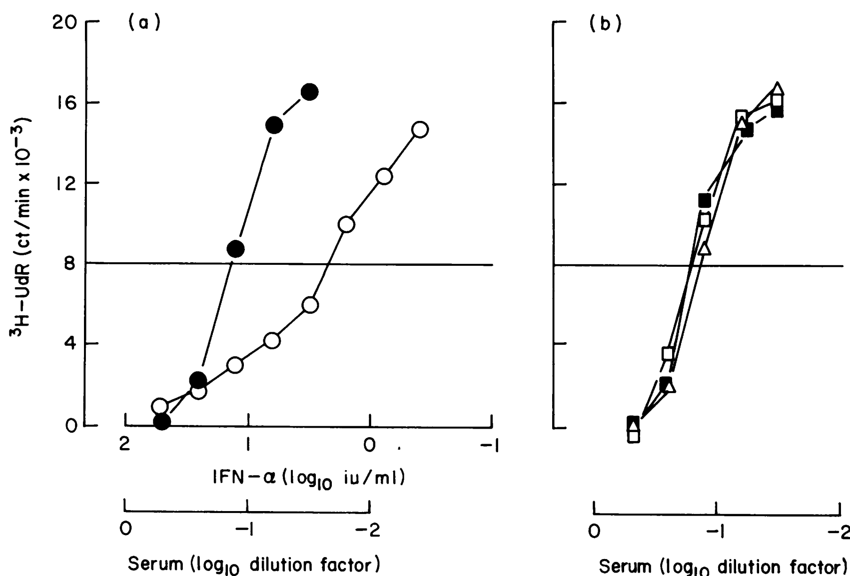

Fig. 4. Inhibition of SFV RNA synthesis in HEp 2 cells by high concentrations of human serum and the failure of antisera against IFN-α, IFN-β or IFN-γ to neutralise the effect. 50% inhibition of viral RNA synthesis is marked by a continuous line. (a) MRC 69/19 human leucocyte IFN standard = ○; human serum obtained from a healthy donor = ●. (b) Human serum as in (a) after incubation with antisera against IFN-α = △; IFN-β = ■; IFN-γ = □. Antisera were diluted sufficiently to neutralize 100 units of IFN-α, β and γ respectively.

Effect of glucocorticosteroid therapy on IFN responses

Nine out of 18 systemic JCA patients received prednisolone during the study but this treatment had no significant effect ($P > 0{\cdot}05$) on *in vitro* IFN responses (results not shown).

Serum IFN

Human serum at high concentrations inhibited SFV RNA synthesis in EBTr and HEp 2 cell lines. This is illustrated in Fig. 4a for HEp 2 cells incubated with normal human serum before the addition of SFV. However, the dose–response curve for human serum differs from that of the IFN-α standard. In the same experiment, antisera to Hu IFN-α, Hu IFN-β or Hu IFN-γ were unable to neutralize the anti-viral activity of serum (Fig. 4b), therefore to avoid this non-specific antiviral effect of high serum concentrations, serial dilutions were routinely prepared from a starting dilution of 1/10. No IFN was detected in any serum sample on either cell line (IFN-α < 10 iu/ml; IFN-γ < 50 u/ml). The sensitivity of the immunoradiometric assay was poor (80 iu/ml) and none of the sera were positive.

DISCUSSION

Our findings suggest that the IFN system in patients with systemic JCA is not grossly abnormal although MNC from these patients may produce more IFN-α in comparison with MNC from control children particularly during a systemic flare. Sequential IFN-α responses of patients' MNC to NDV fluctuated (Fig. 2a); in some patients responses varied over a range of 3 $\log_{10}$ iu/ml. As culture conditions were carefully standardized, differences in the IFN-α response of MNC from a given patient presumably reflect differences in the cell population cultured *in vitro*. Ideally, sequential IFN responses of MNC from control children should have been assessed but this was not practical.

MNC from patients with systemic flare produced more IFN-α in response to NDV than at times when the same patients were clinically stable. Perhaps during a systemic flare the proportion or activity of responding peripheral blood MNC is increased. It would be of interest to extend our study to IFN-α inducers other than NDV since patients' MNC may be hyperresponsive to a range of inducers. In the mouse it has been observed that different genes quantitatively influence responses to different IFN inducers (de Maeyer & de Maeyer-Guignard, 1979).

No significant differences were found between patient and control groups when maximum *in vitro* IFN-γ responses were compared (Fig. 1b) or when responses at each PHA dose were compared (results not shown). Although some variation in sequential IFN-γ responses of patients' MNC was apparent (Fig. 2b) the range was $< 1{\cdot}5$ $\log_{10}$ u/ml.

Seven cultures (four control, three patient) failed to respond to NDV but IFN-γ was always produced in parallel cultures stimulated with PHA. Similarly, six cultures from patients failed to produce IFN-γ but parallel cultures always produced IFN-α in response to NDV, therefore poor cell viability is unlikely to explain these results. MNC from the youngest patient, J.Y., never produced IFN-γ. The age of J.Y. might account for the deficiency since Bryson *et al.* (1980) reported that MNC from neonates proliferated in response to PHA but failed to produce IFN-γ. Farrant *et al.* (1980) suggested that in order to assess proliferative lymphocyte responses *in vitro*, cell concentration, mitogen dose and time of culture need to be considered together. These variables may also interact to control the IFN-γ response but in a manner distinct from the proliferative response.

Our results suggested that high serum concentrations inhibited SFV RNA synthesis and that this activity was unrelated to IFN (Fig. 4). By mixing normal human serum with known amounts of IFN-α we verified that IFN could be detected in serum at concentrations $\geqslant 10$ iu/ml on EBTr cells (unpublished observations). However, serum IFN was not detected in any patient or control in contrast with results from other investigations of connective tissue disease, some of which correlate disease activity with serum IFN titres in patients with systemic lupus erythematosus (SLE; Hooks *et al.*, 1979; Ytterberg & Schnitzer, 1982). It is not clear whether this discrepancy reflects pathogenetic differences between systemic JCA and SLE or differences in methodology since IFN assays used in

these other studies have been based on the inhibition of virus-induced cytopathic changes in cell monolayers.

During our study of patients with systemic JCA the majority of clinical infections involved the upper respiratory tract (de Vere-Tyndall *et al.*, 1983). Failure to detect serum IFN during or shortly after a clinical infection could indicate that IFN production was localized or only transient. Detectable levels of IFN would not persist *in vivo* as IFN-α disappears from serum within 24 h after intramuscular injection into volunteers (Scott *et al.*, 1981) and more rapidly after intravenous injection (Strander *et al.*, 1973).

Because most flares in our patients were preceded by a clinical infection (de Vere-Tyndall *et al.*, 1983) the possibility that enhanced levels of IFN-α generated during an infection contribute to the pathogenesis of systemic JCA merits consideration. Two areas of research indicate that IFN is not necessarily beneficial to the host. First, IFN induction and disease severity in mice infected with lymphocytic choriomeningitis virus are related (Rivière *et al.*, 1977, 1980; Jacobson, Friedman & Pfau, 1981), and secondly, the autoimmune disease in NZB/NZW F_1 hybrid mice is accelerated by exogenous IFN (Steinberg, Baron & Talal, 1969; Engleman *et al.*, 1981). Prostaglandins are important mediators of inflammation (Bonta & Parnham, 1978) and are induced in response to IFN (Yaron *et al.*, 1977; Fitzpatrick & Stringfellow, 1980). Perhaps enhanced synthesis of IFN-α by patients with systemic JCA during an infection leads to overproduction of prostaglandins which contribute to an exacerbation of the underlying disease.

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REFERENCES

ANSELL, B.M. (1978) Chronic arthritis in childhood. *Ann. Rheum. Dis.* **37,** 107.

BONTA, I.L. & PARNHAM, M.J. (1978) Prostaglandins and chronic inflammation. *Biochem. Pharmacol.* **27,** 1611.

BRYSON, Y.J., WINTER, H.S., GARD, S.E., FISCHER, T.J. & STIEHM, E.R. (1980) Deficiency of immune interferon production by leukocytes of normal newborns. *Cell Immunol.* **55,** 191.

DE MAEYER, E. & DE MAEYER-GUIGNARD, J. (1979) Considerations on mouse genes influencing interferon production and action. In *Interferon I* (ed. by I. Gresser) p. 75. Academic Press, New York.

DE VERE-TYNDALL, A., BACON, T., PARRY, R., TYRRELL, D.A.J., DENMAN, A.M. & ANSELL, B.M. (1983) Infection and interferon production in systemic juvenile chronic arthritis—a prospective study. *Ann. Rheum. Dis.* (In press.)

ENGLEMAN, E.G., SONNENFELD, G., DAUPHINEE, M., GREENSPAN, J.S., TALAL, N., MCDEVITT, H.O. & MERIGAN, T.C. (1981) Treatment of NZB/NZW F_1 hybrid mice with *Mycobacterium bovis* strain BCG or type II interferon preparations accelerates autoimmune disease. *Arthrit. Rheum.* **24,** 1396.

EULAR BULLETIN NO. 4 (1977) *Nomenclature and classification of arthritis in children* (ed. by E. Munthe) National Zeitung A.G., Basel.

FARRANT, J., CLARK, J.C., LEE, H., KNIGHT, S.C. & O'BRIEN, J. (1980) Conditions for measuring DNA synthesis in PHA stimulated human lymphocytes in 20 μl hanging drops with various cell concentrations and periods of culture. *J. Immunol. Meth.* **33,** 301.

FITZPATRICK, F.A. & STRINGFELLOW, D.A. (1980) Virus and interferon effects on cellular prostaglandin biosynthesis. *J. Immunol.* **125,** 431.

HOOKS, J.J., MOUTSOPOULOS, H.M., GEIS, S., STAHL, N.I., DECKER, J.L. & NOTKINS, A.L. (1979) Immune interferon in the circulation of patients with autoimmune disease. *N. Engl. J. Med.* **301,** 5.

HOOKS, J.J., JORDAN, G.W., CUPPS, T., MOUTSOPOULOS, H., FAUCI, A.S. & NOTKINS, A.L. (1982) Multiple interferons in the circulation of patients with systemic lupus erythematosus and vasculitis. *Arthrit. Rheum.* **25,** 396.

JACOBSON, S., FRIEDMAN, R.M. & PFAU, C.J. (1981) Interferon induction of lymphocytic choriomeningitis viruses correlates with maximum virulence. *J. gen. Virol.* **57,** 275.

KOBLET, H., KOHLER, U. & WYLER, R. (1972) Optimization of the interferon assay using inhibition of Semliki Forest virus-ribonucleic acid synthesis. *Appl. Microbiol.* **24,** 323.

MCWILLIAMS, M., FINKELSTEIN, M.S., ALLEN, P.T. & GIRON, D.J. (1971) Assay of chick interferon by the inhibition of viral ribonucleic acid synthesis. *Appl. Microbiol.* **21,** 959.

OHNO, S., KATO, F., MATSUDA, H., FUJII, N. & MINAGAWA, T. (1982) Detection of gamma interferon in the sera of patients with Behçet's disease. *Infect. Immun.* **36,** 202.

PREBLE, O.T., BLACK, R.J., FRIEDMAN, R.M., KLIPPEL, J.H. & VILCEK, J. (1982) Systemic lupus erythematosus: presence in serum of an unusual acid-labile leukocyte interferon. *Science*, **216,** 429.

RIVIÈRE, Y., GRESSER, I., GUILLON, J.-C. & TOVEY,

M.G. (1977) Inhibition by anti-interferon serum of lymphocytic choriomeningitis virus disease in suckling mice. *Proc. Natl. Acad. Sci. USA*. **74,** 2135.

RIVIÈRE, Y., GRESSER, I., GUILLON, J.-C., BANDU, M.-T., RONCO, P., MOREL-MAROGER, L. & VERROUST, P. (1980) Severity of lymphocytic choriomeningitis virus disease in different strains of suckling mice correlates with increasing amounts of endogenous interferon. *J. exp. Med.* **152,** 633.

SCOTT, G.M., SECHER, D.S., FLOWERS, D., BATE, J., CANTELL, K. & TYRRELL, D.A.J. (1982) Toxicity of interferon. *Br. Med. J.* **282,** 1345.

STEINBERG, A.D., BARON, S. & TALAL, N. (1969) The pathogenesis of autoimmunity in New Zealand mice. I. Induction of antinucleic acid antibodies by polyinosinic polycytidilic acid. *Proc. Natl. Acad. Sci. USA*. **63,** 1102.

STEWART, W.E. (1979) Varied biologic effects of interferon. In *Interferon 1* (ed. by I. Gresser) p. 29. Academic Press, New York.

STRANDER, H., CANTELL, K., CARLSTRÖM, G. & JAKOBSSON, P.A. (1973) Clinical and laboratory investigations on man: systemic administration of potent interferon to man. *J. Natl. Cancer Inst.* **51,** 733.

SUZUKI, J., AKABOSHI, T. & KOBAYASHI, S. (1974) A rapid and simple method for assaying interferon. *Jap. J. Microbiol.* **18,** 449.

YARON, M., YARON, I., GUARARI-ROTMAN, D., REVEL, M., LINDNER, H.R. & ZOR, U. (1977) Stimulation of prostaglandin E production in cultured human fibroblasts by poly(I)–poly(C) and human interferon. *Nature*, **267,** 457.

YTTERBERG, S.R. & SCHNITZER, T.J. (1982) Serum interferon levels in patients with systemic lupus erythematosus. *Arthrit. Rheum.* **25,** 401.