Human Leukocyte Interferon: The Variation in Normals and Correlation with PHA Transformation[1] (36655)

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The demonstration that human peripheral blood leukocytes are capable of producing interferon (IF) *in vitro* (1–3) has added a new parameter for the *in vitro* assessment of cellular immune mechanisms. Evidence has been presented from this laboratory, and others, that the leukocytes in certain disease states may be defective in IF production (4–9). As a part of these investigations, we have studied 52 leukocyte cultures from 28 normal individuals on 35 separate occasions in an attempt to determine the range of normal response, the reproducibility of this response for any given individual, and the relationship between the IF titer, the white blood cell differential count, and phytohemagglutinin (PHA) induced blastic transformation.

Materials and Methods. Leukocyte cultures. Leukocyte-rich plasma was obtained by gravity sedimentation of peripheral blood samples anticoagulated with 30–100 units/ml of preservative free heparin (Panheprin, Abbott Laboratories, North Chicago, IL) obtained from 28 donors (19 males, 9 females), ages 20 to 60. All donors were free of symptoms of viral disease and had ingested no drugs for at least 12 hr before study. The cells were centrifuged at 150*g* and washed twice with TC 199 + 20% fetal calf serum supplemented with 100 units penicillin/ml to remove the plasma and platelets (10).

IF production. Two ml of culture medium containing 2×10^6 cells/ml, and Newcastle disease virus (NDV) at multiplicities of infection (m.o.i.) ranging from 0.1 to 5.0 plaque forming units (PFU)/leukocyte, were incubated at 37° in a 5% CO_2 atmosphere for 24 hr. To the culture supernatants, 1.0 *N* HCl was added to acidify to pH 2.0, and the samples were incubated for 48 hr at 4° to inactivate residual NDV. The supernatants were then returned to pH 7.0 and were stored at −70°. NDV plaques could not be demonstrated on chick embryo monolayers after such treatment. The virus inhibitory substance obtained satisfied the criteria for an interferon (11).

PHA transformation. A 2 ml volume containing 1×10^6 leukocytes and 0.01 ml PHA prepared according to the method of Börjeson *et al.* (12), was incubated for 96 hr at which time a 4 hr pulse of 1 μCi ^{3}HTdr was given, and the uptake of the isotope into DNA was measured as described previously (13). The results are expressed as disintegrations per minute (dpm)/1×10^6 leukocytes.

IF assay. Samples were diluted in 2-fold steps and assayed by a dye uptake method utilizing human foreskin derived fibroblast monolayers and vesicular stomatitis virus (VSV) (11). All values are expressed in international units (IU) based upon titration of international reference IF 69/19 in our assay system. Simultaneous titration of 6 separate dilutions of a single laboratory reference IF yielded close agreement with a coefficient of variation of 0.11.

Virus. NDV, C_G strain, grown in chick allantoic fluid, and VSV, Indiana serotype, grown in human fibroblast monolayers, were titered (PFU/ml) on chick embryo and human fibroblast layers, respectively.

Results. Conditions of IF production. In preliminary experiments, incubation mixtures containing $2–5 \times 10^6$ leukocytes/ml were found to yield high IF titers. A significantly

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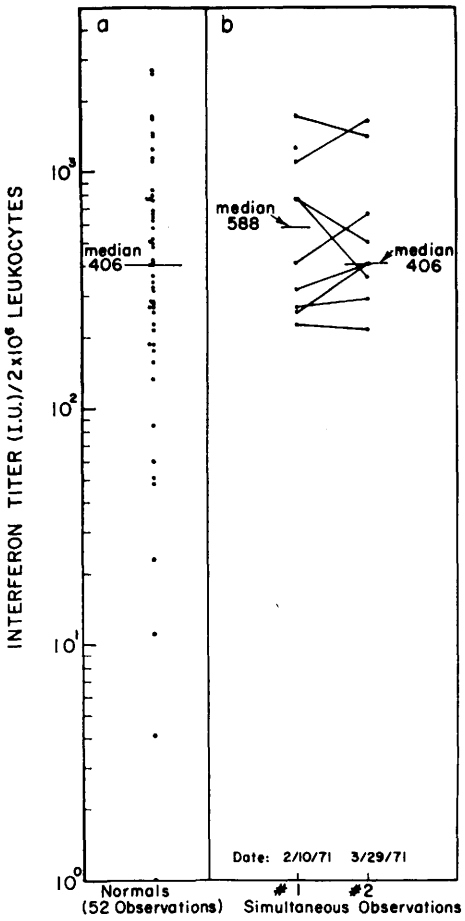

FIG.1a. The leukocyte interferon response of normals. (b) The interferon response of 10 normal individuals studied concurrently and of 9 of the 10 studied on a second occasion. Values for the same individual are connected.

inferior titer was seen at concentrations of 1 $\times$ 10^6 cells/ml or lower. A concentration of 2 $\times$ 10^6 leukocytes/ml was consequently chosen for all studies. Investigation of the kinetics of leukocyte IF production confirmed reports that all of the IF appeared in the supernatant by 16–20 hr and that NDV replication could not be demonstrated during incubation (3).

IF response. When the leukocytes from normal individuals were infected with NDV at an m.o.i. of 0.1 PFU/leukocyte, a wide range of IF response was seen, varying from a low of $<$ 0.7 IU to a high of 2654 IU, with a median of 406 IU (Fig. 1a). There was no signifiant difference in the IF response between males and females, nor between individuals of different ages. Spontaneous production of antiviral activity in control leukocyte cultures incubated without virus was not detected ($<$ 0.04 to $<$ 14.4 IU; median $<$ 2.0 IU).

Variability of the IF response. In an effort to explain the observed variability of IF production, technical factors were examined. The cell washing procedure had eliminated possible factors present in autologous plasma. Fetal calf serum was purchased in large batches, and aliquots of the same lot were used over periods during which both high and low IF titers were obtained. Most of the results had been obtained from culturing the cells of only one or two normal individuals simultaneously, thus subtle differences in media, cell handling techniques, conditions of incubation, and the IF assay might explain the variability. This possibility was tested by simultaneously culturing the leukocytes from 10 normal individuals on the same day. Nine of the same 10 were studied a second time 7 weeks later. Each set of supernatants were then assayed in the same experiment.

TABLE I. Tube to Tube Variability of IF Production.

IF titer, sample no. 1	Mean	Coef. of variation	IF titer, sample no. 2	Mean	Coef. of variation
507[a]	562	0.18	676[a]	789	0.20
635			730		
487			811		
460			987		
717			946		
568			581		

[a] Pooled supernatant from 5 tubes.

The results (Fig. 1b) again show considerable variation in the IF response between individuals and a median not significantly different from that of all the subjects studied.

Biological factors as an explanation for variability were also examined. In two experiments, 10 replicate leukocyte culture tubes were prepared. After incubation, the supernatants of 5 were pooled, and the remaining 5 were processed separately. All 6 samples of each IF were assayed on the same day (Table I). A difference in interferon production between replicate tubes is seen. This probably reflects tube to tube biological variability, since the coefficients of variation, 0.18 and 0.20, respectively, are in excess of that seen when 6 aliquots of a single IF sample were assayed stimultaneously (0.11). This difference, however, is by no means large enough to explain the much greater differences seen between individuals.

Whole leukocyte cultures contain a heterogeneous population of cells, of which only mononuclear cells are believed to produce IF in response to virus challenge (2, 3). Consequently the data were examined to see if any correlation existed between the percentage of mononuclear cells in the culture and the amount of IF produced (Fig. 2). No correlation could be found.

Correlation of the IF response with PHA response. Since the small lymphocyte plays a central role in the process of cellular immunity, it might be expected that the reactivity of the lymphocyte as measured by PHA induced transformation would be related to the reactivity as determined by the release of an effector substance. Figure 3 compares the PHA induced transformation with the NDV induced IF response in 36 cultures. No correlation was seen.

Effect of virus dose on IF production. In 17 subjects, a dose response curve of IF production by NDV was run at 3 or more virus concentrations in an effort to determine the optimal virus dose, and to see if a maximal IF response could be elicited which was more uniform that that obtained at a single virus dose. In 12 subjects, the maximal IF titer was obtained at a m.o.i. of 0.1 PFU/leukocyte. Higher virus doses (1.0 and 5.0) resulted in the same, or often significantly lower, IF titers. In 5 individuals, 1.0 PFU/leukocyte was optimal. Low IF titers were always observed at a m.o.i. of 0.01 PFU/leukocyte.

Similar findings were observed in 6 additional subjects studied at 2 different virus

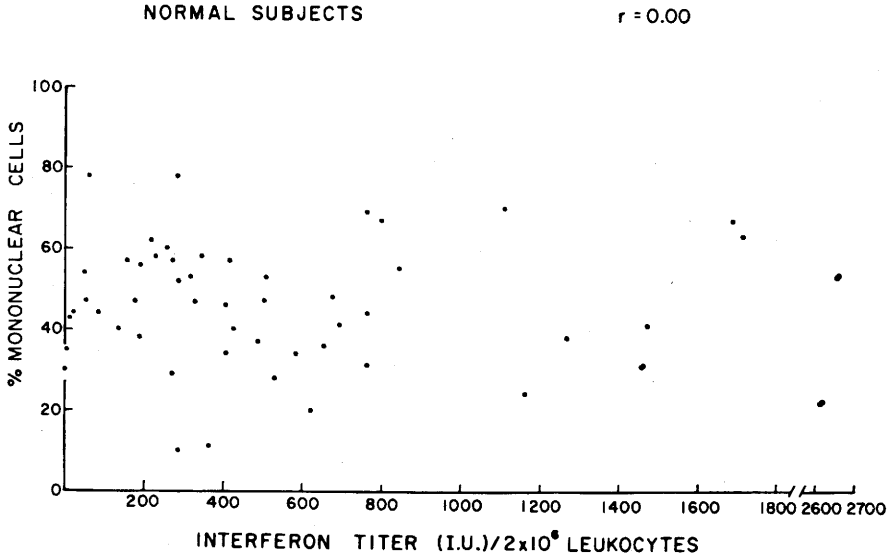

FIG. 2. The relationship of the IF titer to the proportion of mononuclear cells in the leukocyte culture.

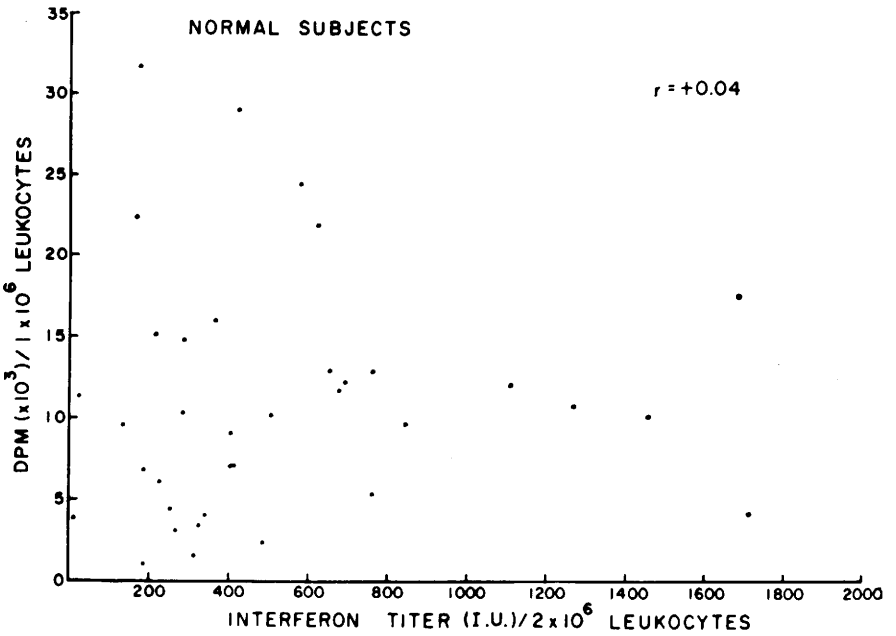

FIG. 3. The relationship between the IF titer and the PHA transformation.

doses. The leukocytes of 2 subjects produced more IF at 0.1 than at 1.0 PFU/leukocyte. The remaining 4 produced the same amount of IF at both doses. This latter group included 2 normal, healthy subjects whose leukocytes did not produce IF in response to NDV either at a dose of 0.1 PFU/leukocyte ($<$ 0.7 IU, $<$ 4.2 IU) or 1.0 (3.4 IU, $<$ 10.1 IU).

Since we found that the IF titers produced when higher doses of NDV were employed were often significantly lower than the titer produced at a lower m.o.i., the role of the concentration of egg albumin, with which the NDV was associated, was investigated. A virus dose of 0.1 PFU/leukocyte resulted in a final concentration of approximately 2% egg albumin. Normal egg albumin (BUN, 6 mg/100 ml; creatinine 0.4, mg/100 ml) was added to leukocyte cultures challenged with varying doses of NDV and did not affect the amount of IF produced.

The variation of the maximal NDV induced IF titer in these 23 cultures, studied at 2 or more virus doses (3.4 to 2615 IU; median 412 IU), was as great as that which had been observed in the 52 samples studied at one virus dose, and no better correlation was seen between IF response and cell differential or PHA transformation.

Day to day variation for the same individual. The ability of the leukocytes of 2 normal individuals to produce IF was studied on repeated occasions over an 18 month period (Fig. 4a and b). Both subjects show a marked variation in IF production. It is clear that this variation was not related to changes in the proportion of mononuclear cells in the culture and did not correlate with the simultaneously determined PHA transformation.

Discussion. The present experiments indicate that the variability in the IF response of human peripheral leukocytes to virus infection *in vitro* is due to intrinsic differences within the leukocytes. Although it is known that the presence of undefined factors in autologous plasma alter the response of leukocytes to PHA (10), and that uremic plasma suppresses the production of IF by normal lymphocytes (8), such factors are unlikely to account for our results as the cells studied were carefully washed before culturing. Washing procedures, of course, may not remove inhibitory substances which may have been previously bound to the leukocyte

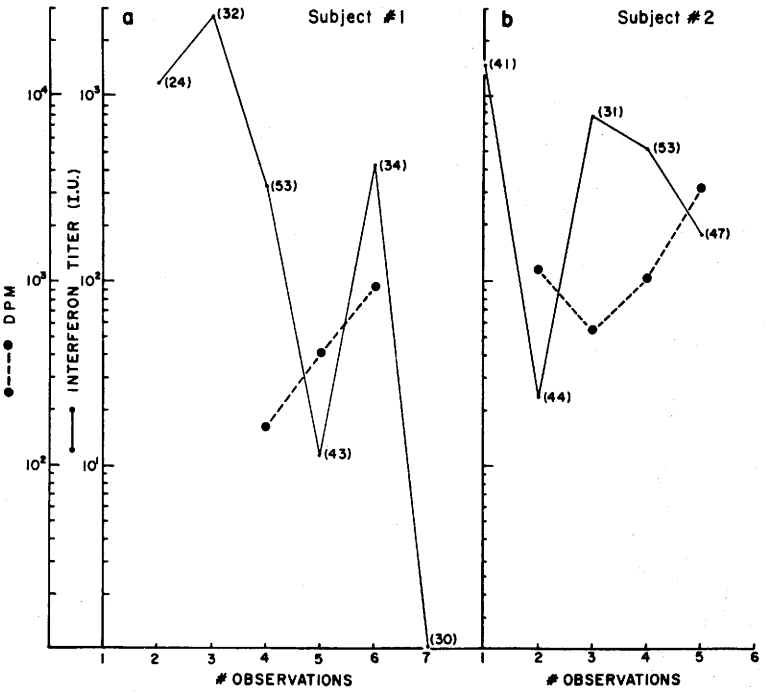

FIG. 4. The variability of the IF and PHA response over an 18 month period for 2 normal individuals. The percentage of mononuclear cells in the differential count is given at each point.

in vivo. The observation that marked variability in IF production occurred when 10 subjects were studied concurrently suggests that technical factors cannot explain such differences between individuals. Furthermore, the marked variation in leukocyte IF response in the same individual when studied repeatedly over a long period of time lends support to our thesis that intrinsic differences exist in the capability of the leukocyte to produce IF in response to an *in vitro* virus infection.

Unlike the findings of Strander *et al.* (9), no correlation was found between the proportion of mononuclear cells in the culture and the IF titer. Thus the problem as to how to interpret an IF titer derived from a leukocyte culture of an individual with a relative or absolute lymphopenia remains. Preparation of purified lymphocyte cultures by filtration of the leukocytes through a glass bead or nylon fiber column does not provide an ideal system to answer this question, as the presence of only small numbers of glass adherent cells greatly enhance the PHA responsiveness of lymphocytes (14). Similarly, a lymphocyte–monocyte interaction in the production of IF has been clearly demonstrated for PHA induced IF (15) and has been suggested to occur in virus induced IF (2). Use of other methods which achieve a removal of granulocytes with the preservation of monocytes and lymphocytes may resolve this question.

The lack of correlation between the NDV induced IF response and the PHA response is similar to that described by Epstein, Cline and Merigan (15) for PHA induced IF. Virus interaction with the leukocyte results in IF production through the synthesis first of messenger RNA and then of newly formed IF (16). This reaction occurs rapidly and is terminated within 24 hr. PHA, on the other hand, initiates a complex sequence of biochemical events terminating in DNA synthesis and cell division 48 hr later (17). Cell membrane interactions are probably required for the initiation of both reactions, but it is likely that different receptor sites are involved. Instances of dissociation between

transformation and the elaboration of effector substances have been described (18). Our data show instances where defective IF production occurred, despite normal PHA transformation. The converse was also observed, most strikingly in 2 patients suffering from acute gastroenteritis who had total suppression of their normal PHA response, but normal IF production (unpublished observation).

The significance of an occasional observation of extremely low IF production in a normal individual is unknown. Such observations were accepted as valid only if other individuals studied concurrently had normal PHA or IF responses, thus making it unlikely that technical factors were the explanation. All individuals studied with extremely low IF responses showed a normal IF response on other occasions. It is possible that these subjects were studied at a time when their leukocytes were in the refractory period following a subclinical virus infection.

This transient state of human leukocyte hyporesponsiveness to *in vitro* induction of IF by virus has not previously been reported. This phenomenon, however, has been clearly demonstrated in *in vitro* studies, where human leukocytes, preincubated with virus or other IF inducers, were rendered hyporesponsive to subsequent virus infection (19). Furthermore, considerable *in vivo* animal data demonstrate a refractory period following administration of an IF inducer, during which time a second IF inducer is usually less effective in producing IF (20). This refractory state, however, is not detrimental to the animal, as a high resistance of virus infection is maintained during this period.

Spontaneous IF production has been observed in some continuous human lymphoblastoid cell lines (21). Consequently, the control cultures from subjects demonstrating defective leukocyte IF production were examined to see if spontaneous IF production or ^{3}HTdr incorporation occurred. No evidence for this was found.

It remains speculative as to how these marked alterations in leukocyte IF production relate to the manner in which the individual copes with a virus infection. If the observed instances of low IF production do, in fact, represent a postviral refractory state, resistance to virus infection *in vivo* may be maintained. However, if these fluctuations reflect *in vivo* lapses in leukocyte reactivity, individuals may have periods of increased susceptibility to the proliferation of some viruses. The possibility of transient periods of lapses in immunological surveillance exists. If so, the lack of correlation between the PHA response and IF production, however, suggests that such lapses may affect only one lymphocyte function at a time.

Summary. Fifty-two leukocyte cultures obtained from 28 healthy, normal individuals studied on 35 separate occasions produced a wide range of interferon titers (IF) in response to infection with Newcastle disease virus (NDV) *in vitro* ($<$ 0.7 to 2654 IU; median 406 IU). This variability could not be correlated with the proportion of mononuclear cells in the culture nor was any correlation noted between the IF titer and simultaneously determined phytohemagglutinin (PHA) blastic transformation. Technical factors could not explain these findings as a similar variability was noted when 10 individuals were studied concurrently. Repeated studies of 2 individuals over 18 months showed marked differences in the IF produced by their leukocytes on these separate occasions. Several subjects were encountered whose leukocytes produced no IF on one occasion, but were responsive to NDV on follow-up study. Spontaneous production of IF by control leukocyte cultures without virus by these individuals, and the group as a whole, was not found. The biological significance of such periods of transient leukocyte hyporesponsiveness to *in vitro* virus infection is unknown.

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1. Friedman, R. M., and Cooper, H. L., Proc. Soc. Exp. Biol. Med. **125**, 901 (1967).

2. Lee, S. H. S., and Ozere, R. L., Proc. Soc. Exp. Biol. Med. **118**, 190 (1965).

3. Wheelock, E. F., J. Bacteriol. **92**, 1415 (1966).

4. Hadhazy, G., Gergely, L., Toth, F. D., and Szegedi, G., Acta Microbiol. Acad. Sci. Hung. **14**, 391

(1967).

5. Lee, S. H. S., van Rooyen, C. E., and Ozere, R. L., Cancer Res. **29**, 645 (1969).

6. Pidot, A. L. R., and McIntyre, O. R., Clin. Res. **19**, 495 (1971).

7. Ray, C. G., and Davis, S. D., Lancet **1**, 312 (1970).

8. Sanders, C. V., Jr., Luby, J. P., Sanford, J. P., and Hull, A. R., J. Lab. Clin. Med. **77**, 768 (1971).

9. Strander, H., Cantell, K., Leisti, J., and Nikkila, E., Clin. Exp. Immunol. **6**, 263 (1970).

10. McIntyre, O. R., and Cole, A. F., Int. Arch. Allergy Appl. Immunol. **35**, 105 (1969).

11. Pidot, A. L. R., Appl. Microbiol. **22**, 671 (1971).

12. Börjeson, J., Bouveng, R., Gardell, S., Norden, A., and Thunell, S., Biochim. Biophys. Acta **82**, 158 (1964).

13. Maurer, L. H., Andrews, P., Rueckert, F., and McIntyre, O. R., Plast. Reconstr. Surg. **46**, 458 (1970).

14. Levis, W. R., and Robbins, J. H., Exp. Cell Res. **61**, 153 (1970).

15. Epstein, L. B., Cline, M. J., and Merigan, T. C., *in* "Fifth Leukocyte Culture Conference" (Jules E. Harris, ed.), p. 501. Academic Press, New York (1970).

16. Lee, S. H. S., Appl. Microbiol. **18**, 731 (1969).

17. McIntyre, O. R., and Ebaugh, F. G., Jr., Blood **19**, 443 (1962).

18. Rocklin, R. E., Reardon, G., Sheffer, A., Churchill, W. H., and David, J. R., *in* "Fifth Leukocyte Culture Conference" (Jules E. Harris, ed.), p. 639. Academic Press, New York (1970).

19. Strander, H., and Cantell, K., Ann. Med. Exp. Biol. Fenn. **45**, 20 (1967).

20. Ho, M., Breinig, M. K., Postic, B., and Armstrong, J., Ann. N.Y. Acad. Sci. **173**, 680 (1970).

21. Haase, A. T., Johnson, J. S., Kasel, J. A., Margolis, S., and Levy, H. B., Proc. Soc. Exp. Biol. Med. **133**, 1076 (1970).

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