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Mechanisms of macrophage activation in rheumatoid arthritis: the role of gamma-interferon

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SUMMARY

Gamma-interferon (γ-IFN) is a potent inducer of surface expression of class II MHC molecules *in vitro*. Enhanced HLA-DR expression is a characteristic immuno-histological feature of rheumatoid joints. To assess the possible relevance of γ-IFN to macrophage (Mϕ) activation in rheumatoid arthritis (RA) we investigated the spontaneous and mitogen-induced production of γ-IFN by RA lymphocytes using a sensitive radioimmunoassay. Synovial fluids (SF) from a variety of inflammatory and non-inflammatory rheumatic diseases did not contain measurable amounts of IFN. RA lymphocytes from peripheral blood (PBL) and joints failed to show spontaneous γ-IFN production. RA and control PBL were equally responsive to both mitogen stimulation and to the addition of exogenous interleukin 2 (IL-2) as control PBL. SF lymphocytes from RA patients showed a significantly decreased PHA-stimulated γ-IFN production and this was in contrast to the SF lymphocytes from patients with other inflammatory joint diseases who showed significantly increased γ-IFN production compared with matched PB lymphocytes. These results show that γ-IFN production by peripheral blood and joint cells from patients with RA is normal and it remains to be established whether γ-IFN is the factor responsible for the macrophage activation seen in the disease.

Keywords γ-interferon macrophage activation rheumatoid arthritis synovial fluid-lymphocytes

INTRODUCTION

Antigen presentation to human T lymphocytes is restricted by class II MHC molecules (whether DP, DQ or DR) on the surface of antigen presenting cells which can be macrophages (Mϕ), mouse fibroblasts in transfection experiments or on endocrine cells in auto-immune disease such as thyroiditis. In the latter situation it is believed that gamma-interferon (γ-IFN) a T cell product, may be the factor responsible for up-regulating class II expression on thyroid cells (Bottazzo *et al.*, 1983). Considerable *in vitro* evidence has accumulated concerning the potent regulatory properties of γ-IFN on surface class II molecule expression which has been shown to enhance HLA-DR synthesis and expression on human melanoma cell lines (Basham & Merigan, 1983), human monocyte lines (Virilizier *et al.*, 1984) and has been shown to activate class II gene expression in human endothelial cells and fibroblasts (Collins *et al.*, 1984), cells which may have an immune accessory function. In addition, γ-IFN appears to have a preferential enhancing effort on DQ genes of the class II region (Ameglio *et al.*, 1983) the part of the MHC complex more directly analagous to the Ia region of the

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murine system. γ-IFN also activates other aspects of Mϕ function, including the release of interleukin 1 (IL-1) (Arenzana-Seisdedos & Virilizier, 1983) and hydrogen peroxide.

In rheumatoid arthritis (RA) the mechanisms for enhanced and persistent immune responsiveness remain unclear. We have proposed that a possible chronicity mechanism could be the presence of an ongoing autologous mixed lymphocyte reaction (AMLR) in the joint generating lymphokines, including γ-IFN, which could activate Mϕ locally (Janossy *et al.*, 1981). There is abundant evidence for Mϕ activation in RA as shown by increased numbers of DR + Mϕ in the RA synovial membrane (Janossy *et al.*, 1981, Duke, Panayi & Janossy, 1982), excessive DR expression in RA by T cells of the peripheral blood and synovial fluid (Duke *et al.*, 1983) and excessive IL-1 production (Fontana *et al.*, 1982, Nouri, Panayi & Goodman, 1984). These phenomena could be due to γ-IFN production by activated T cells in the joint and the presence of interferon physicochemically resembling γ-IFN in the sera and synovial fluids (SF) of some patients with RA was cited as preliminary evidence for this (Hooks *et al.*, 1979; Degre, Mellbye & Jenssen, 1983, Cesario *et al.*, 1983) until it was released that this was an unusual form of α-IFN. However, these results were obtained using a relatively insensitive bioassay (inhibition of virally-induced cytopathic effect) of body fluids which may contain inhibitors or enhancers of such a system. With the availability of sensitive radioimmunoassays for γ-IFN employing monoclonal antibodies (MoAb) we decided to investigate the problem by measuring γ-IFN production of PB and SF mononuclear cells in patients with RA. The hypothesis being tested that Mϕ activation in the rheumatoid joint is due to the release of γ-IFN by *in situ* activated T cells. In the event no evidence for spontaneous γ-IFN production in the joint was found and the mechanism for Mϕ activation in RA remains unknown.

MATERIALS AND METHODS

Subjects. Forty two patients with definite or classical RA (Ropes *et al.*, 1958) were studied. PB was studied in all patients but in addition simultaneous samples of SF were obtained from 11 patients and synovial membrane (SM) from two. There were 19 males and 23 females of mean age 52 years (range 16–80) of which 26 were receiving treatment with non-steroidal anti-inflammatory drugs (NSAID) only and 16 were receiving NSAID plus: sodium aurothiomalate (6), D-penicillamine (6), chloroquine (2) or azathioprine (2). All drug treatment was stable for 3 months prior to study. Paired blood and synovial fluid samples were also obtained from four patients with other inflammatory arthropathies (psoriatic arthritis (2), ankylosing spondylitis (2)) and paired blood and synovial membrane samples from two patients with osteoarthritis.

Control blood samples were obtained from 23 subjects of mean age 49 years (range 24–79), nine males and 14 females of which 19 were patients attending the Rheumatology Outpatient Clinic with non-inflammatory disorders (osteoarthritis, adhesive capsulitis, low back pain) and four healthy volunteer laboratory personnel. Ten of these subjects were taking NSAID.

All samples, apart from those from laboratory personnel, were obtained during diagnostic or therapeutic procedures.

Mononuclear cell Separation. PB mononuclear cells (PBMNC) were separated from heparinized PB by density gradient centrifugation on 'lymphoprep' (Nyegaard, Oslo, Norway) and then washed three times in Hank's balanced salt solution (HBSS) (GIBCO, Grand Island, NY, USA). Heparinized SF samples were diluted with HBSS 1:2 and centrifuged at 700 *g* for 10 min. The cell pellet was carefully resuspended in HBSS and layered onto 'lymphoprep' and then handled in the same way as the PBMNC. Synovial membrane mononuclear cells (SMMNC) were obtained using the method of Abrahamsen *et al.* (1976). Briefly, synovial tissue, after washing in HBSS, was minced and dispensed into universals with collagenase (0·5 mg/ml) (Type 1, Sigma Chemical Co., St Louis, MO, USA) and DNase (0·15 mg/ml) (Type 1, Sigma Chemical Co.) and incubated at 37°C for 1–2 h with agitation. The remaining fragments were discarded and the cells resuspended in RPMI 1640 (GIBCO, Grand Island, NY, USA) with 10% fetal calf serum (FCS) (Seralab, Sussex, UK) and cultured at 37°C in tissue culture flasks (Nunc, Roskilde, Denmark) overnight. The following morning the non-adherent cell suspension was removed by shaking the flasks and then layered onto 'lymphoprep' and handled in the same way as the PBMNC. Finally, all MNC were resuspended in

RPMI 1640 supplemented with 10% FCS, HEPES (to 20 mM) (GIBCO, Grand Island, NY, USA) L-glutamine (to 2 mM) and antibiotics a final concentration of 1×10^6 cells/ml.

γ-IFN production. Cell suspensions were incubated at 37°C in 96 well flat-bottomed microtitre plates (Titertek, Flow Labs, UK) for 72 h. Cultures were set up to assess γ-IFN produced (a) spontaneously (b) in the presence of phytohaemagglutinin (PHA) (Sigma Chemical Co., St Louis, MO, USA) 5 μg/ml, (c) in the presence of 100 u/ml recombinant IL-2 (a generous gift from Biogen, Geneva, Switzerland) and (d) in the presence of PHA 5 μg/ml and IL-2 100 u/ml Preliminary time course and dose-response studies revealed the above conditions to be optimal for γ-IFN production after PHA stimulation (data not shown). Supernatants were harvested after centrifugation and stored at −70°C until assayed.

IFN-assay. All supernatants were assayed in duplicate using an immunoradiometric assay (IRMA) (Boots-Celltech Diagnostics, Slough, UK). Briefly, 200 μl aliquots of supernatant were incubated at room temperature with 50 μl ^{125}I-labelled MoAb antibody for 2 h. Fifty microlitres of sheep anti-γ-IFN antibody coupled to solid phase was then added and the samples agitated for 2 h to to ensure even coating. Unbound antibody was then removed by washing twice using the 'Sucrosep' (Boots Cell Tech) system. The remaining radioactivity bound with the solid phase was counted in a gamma counter. The assay has a minimum detection limit of 1 iu/ml γ-IFN. A standard curve was constructed for each assay using a human γ-IFN standard. The standard deviation of the difference between duplicates was $< 5\%$ of the mean total count in 80% of estimations and never $> 10\%$. Intrasubject variation assessed in one normal subject on three separate occasions after PHA stimulation gave values of 19, 25, and 30 u/ml γ-IFN. This is consistent with the findings of Bacon *et al.* (1983) who found that in contrast to viral induction of α-IFN production, mitogen stimulated γ-IFN production was relatively consistent longitudinally both in normals and their patients with juvenile chronic arthritis.

HLA-DR expression on U937 cells. U937 cells (a human myelomonocytic cell line) were kept in continuous culture in complete RPMI with 10% FCS. γ-IFN enhancement of HLA-DR surface expression was assessed using the method outlined by Virilizier *et al.* (1984). Briefly 1×10^6/ml U937 cells were incubated in presence or absence of γ-IFN for 48 h at 37°C. Subsequently the cells were stained using anti-DR monoclonal antibody (kindly provided by Dr L. Poulter, Royal Free Hospital, London, UK) by indirect immunofluorescence with goat anti-mouse labelled fluorescienisothiocyanate (FITC) as a second layer. Cells were finally resuspended in PBS with 1% Paraformaldehyde and analysed on a flow cytometer.

Statistical methods. γ-IFN responses did not have a normal distribution. Therefore non-parametric methods were used: the Wilcoxon signed rank sum test for paired samples and the Mann-U-Whitney test for other analysis.

RESULTS

Absence of spontaneous γ-IFN production by RA PBL

Spontaneous γ-IFN production in the RA group showed a very similar pattern to that demonstrated in normals (Fig. 1) with only a minority of both groups showing any spontaneous production, normally in low amounts (< 10 u/ml).

Addition of exogenous IL-2 to PBL

Addition of IL-2 did produce a small but significant rise in γ-IFN production from spontaneous levels in paired samples in both RA and controls ($P < 0{\cdot}01$). However, taken as a whole neither group showed a significant increase in γ-IFN above spontaneous levels and there was no significant difference between rheumatoid and control responses.

Addition of PHA and IL-2 to PBL

As expected both groups showed significant rises in γ-IFN production following PHA stimulation ($P < 0{\cdot}01$). However there was no significant difference between RA and control responses to PHA stimulation (Fig. 2). Addition of exogenous IL-2 to the PHA cultures further increased γ-IFN

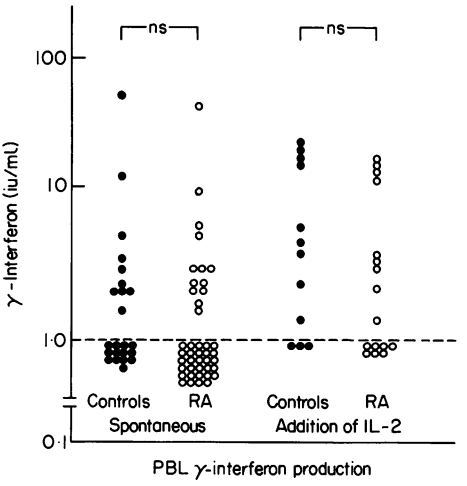

Fig. 1. γ-IFN production by PBL occurring spontaneously and following the addition of rIL-2 100 u/ml. (●) Controls; (○) RA subjects; ns not significant.

production in both groups ($P < 0{\cdot}01$). There was no significant difference in the increased level of γ-IFN production between RA and controls nor was there any correlation between γ-IFN responses and disease activity or duration.

Effect of drugs on γ-IFN responses

There was no significant difference in spontaneous or stimulated γ-IFN production between patients on first- or second-line drugs. Numbers of patients on individual second-line drugs were too small to allow a meaningful analysis of any differences there may have been between individual drugs.

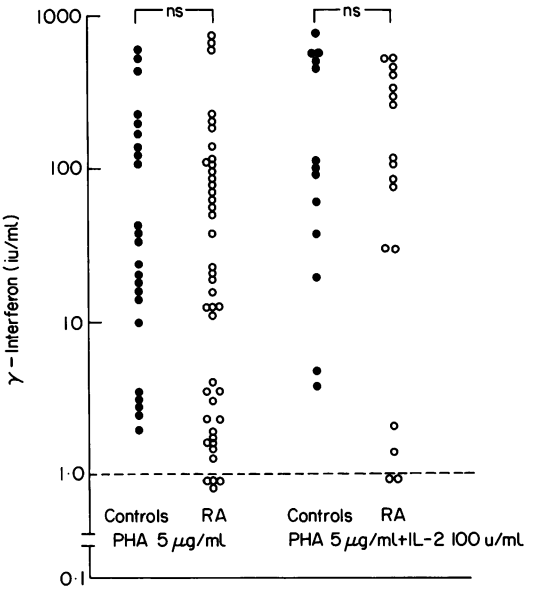

Fig. 2. γ-IFN production by PBL following stimulation with PHA 5 μg/ml or PHA 5 μg/ml + rIL-2 100 u/ml (●) Controls; (○) RA subjects; ns not significant.

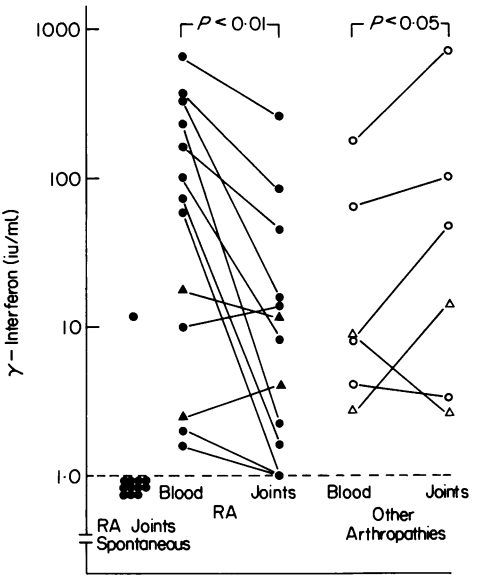

Fig. 3. γ-IFN production after stimulation with PHA 5 μg/ml by paired peripheral blood and joint lymphocytes. (●——●) RA PBL/SFL; (▲——▲) RA PBL/SML; (○——○) other arthropathies PBL/SFL; (△——△) osteoarthritis.

γ-IFN production by joint cells (Fig. 3)

RA synovial fluid lymphocytes (RA SFL) and RA synovial membrane lymphocytes (RA SML) like RA PBL showed no significant spontaneous γ-IFN production. Following PHA stimulation RA SFL produced significantly less γ-IFN ($P < 0{\cdot}01$) than corresponding PBL. Of the two RA synovial membranes one produced more and one less γ-IFN than paired blood samples. When RA SFL and RA SML data was analysed as a group it showed a significant decrease in γ-IFN production compared with paired RA PBL ($P < 0{\cdot}01$). In contrast, SFL from other inflammatory joint diseases (four patients) showed significantly increased γ-IFN production ($P < 0{\cdot}05$) compared with paired PBL. Addition of IL-2 to RA SFL significantly increased γ-IFN production although not to levels comparable with paired RA PBL.

γ-IFN in synovial fluid

Six samples of cell-free centrifuged synovial fluid were tested for γ-IFN activity. None (< 1 u/ml) was detected in any of the samples. Simultaneous assays of standard human γ-IFN added to synovial fluid on the γ-IFN IRMA confirmed that γ-IFN was detectable in synovial fluid using this method (data not shown).

Activation of class II MHC expression by γ-IFN

U937 cells incubated without γ-IFN did not express DR molecules. The addition of as little as 2 u/ml γ-IFN resulted in 10% of cells expressing DR while 20 u/ml increased this to 30%.

DISCUSSION

The main findings of this study are (a) the absence of spontaneous γ-IFN production by RA, PBL, SFL and SML, despite the use of a sensitive radioimmunoassay capable of detecting 1 u/ml γ-IFN, (b) RA PBL shows a profile of stimulated responses very similar to controls, (c) RA SFL produce less γ-IFN in response to mitogen stimulation than paired PBL while, by contrast, in patients with other inflammatory joint diseases there was a tendency for γ-IFN production to be greater in SFL

than in PBL. Addition of exogenous IL-2 resulted in further enhancement of γ-IFN production by both RA and controls.

The absence of *in vitro* spontaneous γ-IFN production by RA lymphocytes and the failure to detect γ-IFN in joint fluids indicates that ongoing γ-IFN production is unlikely to be present in RA. Other workers have suggested that γ-IFN production is defective in RA (Hasler, 1983; Chin, 1983; Combe, 1985). However, they have all used conventional viral inhibition bioassays to measure γ-IFN in which the presence of interferon inhibitors in the biological fluid being assayed could spuriously affect the measurement of functional amounts of IFN. The use of radioimmunoassay avoids this problem and allows a more precise and direct measurement. Our results suggest that γ-IFN production both spontaneously and after PHA stimulation are the same in RA and normals.

There is evidence for defective IL-2 production in the rheumatoid joint despite the presence of activated T cells (Combe *et al.*, 1985, Nouri & Panayi unpublished observations)) and it could be argued that since IL-2 is necessary for optimal production of γ-IFN (Farrar, Johnson & Farrar, 1981, Pearlstein *et al.*, 1983) then this could be a mechanism for the relative deficiency of γ-IFN from joint cells in RA. However, we have shown that the addition of recombinant IL-2 alone did not induce spontaneous γ-IFN production. The increase in PHA stimulated γ-IFN production was particularly marked in the PBMNC but of equal magnitude in RA and control subjects. In the SF γ-IFN production by RA was significantly lower than in corresponding PBL and did not reach PBL levels even in the presence of IL-2, implying some abnormality in the joint compartment.

The precise population of cells subserving γ-IFN production is not yet clear but it appears likely that many of the relevant cells are contained within the 'third population' of lymphocytes (O'Malley *et al.*, 1982) and this population includes natural killer (NK) cells. NK number and function has a wide normal range and is affected by age and sex. However, our groups were well matched for age and had virtually identical sex-ratios so it seems unlikely that this influenced the observed variability of responses. NK cell number is also very variable in RA subjects and indeed one recent study (Faure *et al.*, 1983) demonstrated a much wider range of NK cell number in PB of RA patients using immunofluorescence with HNK-1 MoAb. There was also a tendency for RA subjects to have a higher proportion of HNK-1$^+$ cells. The situation is further complicated by the fact that although HNK-1 defines most of the cells of the NK population it does not appear to specifically define those NK cells which are fully functional. We are currently investigating the possibility that some of the variation in γ-IFN responses between RA patients and between RA PBL and RA SFL is due to different numbers of functional NK cells. In view of the known potent enhancing effects of γ-IFN on NK activity (Weigent, Stanton & Johnson, 1983; Handa *et al.*, 1983), the reduced IFN production by SFL is consistent with earlier observations from our laboratory (Armstrong & Panayi, 1983)) and others (Dobloug *et al.*, 1982; Silver *et al.*, 1982) that NK activity is reduced in the RA joint.

In view of the Mϕ activating properties of γ-IFN, failure to demonstrate significant spontaneous γ-IFN production raises questions as to the mode of Mϕ activation in RA. However, since as little as 2 u/ml γ-IFN is capable of activating Class II MHC expression on U937 cells it is possible that small amounts of locally produced γ-IFN could account for Mϕ activation in the joint. Although, as discussed earlier, γ-IFN is now thought to account for a large proportion of macrophage activating factor (MAF) activity, the possibility remains that other lymphokines are being produced which could activate class II MHC expression on Mϕ. Of interest, is the recently described MAF activity produced by a T cell clone which appears distinct from γ-IFN (Andrew *et al.*, 1984). We are therefore currently investigating the possibility that additional lymphokines are being produced in RA which are capable of inducing DR expression on appropriate indicator cells.

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