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Interferon-gamma potentiates antibody affinity in mice with a genetically controlled defect in affinity maturation

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SUMMARY

Interferon-gamma (IFN-γ), the tetrapeptide tuftsin and the synthetic nonapeptide from interleukin-1 beta (IL-1β) (amino acids 163–171) have previously been shown to act on macrophages and/or T cells and to enhance antibody titres to T cell-dependent antigens. The ability of these immunomodulatory agents to potentiate antibody affinity in addition to antibody titre has been studied in a line of mice that fail to demonstrate normal maturation of antibody affinity (low N/M mice). The results presented here confirm that each of the agents potentiate antibody levels following simultaneous injection with a T cell-dependent antigen but demonstrate that only IFN-γ is able to enhance antibody affinity in these mice. The observation that IFN-γ can enhance both antibody affinity and antibody levels suggests that it could be an important adjuvant for vaccine use.

Keywords interferon-gamma antibody affinity adjuvant action

INTRODUCTION

Several adjuvants have been described primarily on the basis of their ability to increase the titre of antibody toward the antigen but only limited work has assessed the action of adjuvants in enhancing the quality or affinity of the antibody response (Petty & Steward, 1977). Several studies have demonstrated that high affinity antibodies are superior to those of lower affinity in terms of biological effectiveness (Steward & Steensgaard, 1983). Thus the potential to enhance the affinity of the antibody response may well be a more important property of an adjuvant than its ability to increase antibody titre. Furthermore, several lines of evidence suggest that the excessive production of low affinity antibodies, which could be considered as an immunocompromised state, leads to the formation and tissue-localization of injurious antigen–antibody complex (for references see Steward, 1987). Indeed, a line of genetically selected mice that fail to show antibody affinity maturation to T cell-dependent antigens (low affinity non-maturing, low N/M) show a greater incidence of antigen–antibody complex disease than do mice showing normal affinity maturation (Devey *et al.*, 1984). Mice of different inbred strains vary in the affinity of antibody they produce to antigens injected in saline, but these differences can be overcome by injection of antigen in Freund's complete adjuvant (FCA) (Petty, Steward & Soothill, 1972). However, the low NM mice fail to produce high affinity antibody even following injection of antigen in FCA (Devey *et al.*, 1984). This failure is associated with the excessive activity of $CD8^+$ T cells, since following anti-CD8 treatment these mice are capable of producing high affinity antibody (Holland & Steward, 1989). Furthermore, the route of immunization has been shown to play an important role in determining the affinity of antibody produced by low N/M mice because injection via the subcutaneous route results in the production of antibody of significantly higher affinity than when antigen was given intraperitoneally. This may reflect differences in antigen processing and presentation by local antigen-presenting cells.

The work described here was designed to exploit the potential of the low N/M mice as immunocompromised animals and we have investigated the effects of three immunomodulatory agents on antibody affinity in these mice. These were recombinant murine interferon-gamma (IFN-γ), the tetrapeptide thr–lys–pro–arg (tuftsin) and the synthetic nonapeptide representing residues 163–171 of human interleukin-1β (IL-1β). The *in vivo* activity of IL-1 has been shown to be mimicked by this nonapeptide (VQGEESNK) (Antoni *et al.*, 1986; Nencione *et al.*, 1987; Frasca *et al.*, 1988). Each of these substances act on macrophages and/or T cells and have been shown to increase the titre of antibody produced toward T cell-dependent antigens (Tzehoval *et al.*, 1980; Nakamura *et al.*, 1984; Sidman *et al.*, 1984; Nencione *et al.*, 1987). Furthermore, it has been suggested (Nencione *et al.*, 1987) that the 163–171 IL-1 peptide may enhance the response to pneumococcal type III antigen by activating specific helper T cells and by abolishing the effect of suppressor T cells which would normally prevent such activation (Braley-Mullen, 1974).

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We confirm the potentiation of antibody titre following simultaneous administration of these agents with antigen in saline and demonstrate that treatment with IFN-γ also results in a significant enhancement of antibody affinity leading to affinity maturation in low N/M mice.

MATERIALS AND METHODS

Mice

Genetically selected low N/M mice from the 30th generation of breeding (Katz & Steward, 1975; Devey *et al.*, 1984) and at 7–8 weeks of age were used in these studies.

Antigens

Crystalline bovine gammaglobulin (BGG), human serum albumin (HSA) and chicken ovalbumin were purchased from Miles Laboratories, Slough, UK.

DNP–protein conjugates at various hapten–protein ratios were prepared by the reaction of 2,4-dinitrobenzene sulphonate with the proteins (Eisen, Carsten & Belman, 1953).

Adjuvants

Recombinant murine IFN-γ was a kind gift from Dr G. R. Adolf (Boehringer, Vienna, Austria) and had a specific activity of 10^7 U/mg protein and a maximum endotoxin contamination of 0·034 U/mg. Tuftsin (thr–lys–pro–arg) was purchased from Sigma Chemical Co. (St Louis, MO). The immunologically active nonapeptide representing residues 163–171 of human IL-1β (Antoni *et al.*, 1986) was synthesized by solid-phase Fmoc chemistry and purified by HPLC. Purity was established by analytical reversed-phase HPLC, amino acid analysis and fast atom bombardment mass spectrometry.

Immunizations

Mice received four once-weekly injections of 500 μg DNP_{19}–BGG in a total volume of 0·2 ml saline including: (i) 1000, 5000, 10 000 or 30 000 U IFN-γ; (ii) 25 μg tuftsin; or (iii) 100 or 200 mg/kg body weight of the IL-1β peptide.

Groups of control mice received (i) the antigen plus 10 ng lipopolysaccharide (LPS) (Sigma) as a control for possible effects of contaminating LPS in the IFN-γ preparation; and (ii) the antigen plus 100 mg/kg of an unrelated peptide from the fusion protein of measles virus.

All mice were bled from the retro-orbital venous plexus under ether anaesthesia 1 week after the last immunization and at two-weekly intervals thereafter.

Determination of antibody affinity and affinity heterogeneity

The most thermodynamically acceptable method for assessing antibody affinity is equilibrium dialysis (Eisen, 1964). However, this method requires relatively large amounts of partially purified antibody and is thus not appropriate for the assessment of affinity in sequential serum samples from individual mice as required in this study. Accordingly, other approaches to the determination of affinity were used. In one group of experiments, anti-DNP antibody affinity was assessed by a double radioisotope globulin precipitation assay (Gaze, West & Steward, 1973) using ^{125}I-labelled $DNP_{2\cdot5}$–HSA as the antigen. Increasing concentrations of ^{125}I-labelled antigen were incubated for 1 h at 4°C with a fixed volume (10 μl) of a predetermined dilution of antiserum. Complexes of antigen and antibody were precipitated by the addition of polyethylene glycol 6000 (final concentration 7·5%) and antibody-bound and free antigen at equilibrium were determined. Antibody levels (Abt, molar antibody combining sites in 10 μl serum) and affinities (M^{-1}) were calculated from a plot of 1/b (bound antigen) *versus* 1/c (free antigen) using the following modification of the Langmuir adsorption isotherm:

$$\frac{1}{b}=\frac{1}{K}\times\frac{1}{c}\times\frac{1}{Ab_t}+\frac{1}{Ab_t}$$

(K represents the equilibrium constant).

While the globulin precipitation assay provides a reliable approach to affinity measurement, it is not readily able to provide information on the heterogeneity of affinities in a polyclonal serum. Thus in the remaining experiments described, the relative affinity and affinity heterogeneity of the anti-DNP antibodies were assessed by an inhibition enzyme immunoassay (Rath, Stanley & Steward, 1988). Serial half-log dilutions of sera were made on DNP_{10}–ovalbumin-coated polystyrene plates and anti-DNP titres were expressed as log dilution giving an end-point optical density of 0·20 (Lew, 1984). Serial dilutions of DNP–lysine (Sigma) were made on DNP–ovalbumin-coated plates and a selected dilution of each serum that yielded an absorbance of approximately 50% maximum value in the titration assay above (i.e. 0·55–0·65) was added. The percentage inhibition at each inhibitor concentration was calculated and the molar concentrations for 50% inhibition ($I_{0\cdot5}$) were calculated. These values represent 'average affinities' and to control for day-to-day assay variations, were expressed as a ratio of the experimental value over that of a control anti-DNP serum assayed under the same conditions. Thus an increase in antibody affinity is represented as a decrease in the affinity ratio. In the figures, the reciprocal of the ratio is employed so that an increase in this value represents an increase in affinity.

The percentile inhibition contributed by each serial inhibitor concentration was calculated and the values used to analyse affinity heterogeneity by histograms.

Monoclonal antibodies

Monoclonal IgG1 anti-DNP antibodies 49, 51, 53 and 57 were isolated from ascitic fluids of mice injected with the hybridomas described by Stanley, Lew & Steward (1983).

RESULTS

The simultaneous administration of each of the three immunomodulating agents and the DNP–BGG antigen resulted in a marked potentiation of antibody levels compared with controls at all bleeds tested (Fig. 1). However, in contrast to the significant effect of each of the adjuvants on antibody titre, only treatment with IFN-γ resulted in potentiation of antibody affinity (Fig. 2). Antibody affinity maturation was observed in mice receiving both 10 000 and 30 000 U of IFN-γ: however, only in the group receiving 10 000 U was the affinity significantly greater ($P<0\cdot01$) than in the control group receiving the low dose of LPS. It should be emphasized that in the inhibition enzyme immunoassay for affinity small differences in the affinity ratios generated represent large differences in affinity as assessed by well-recognized fluid-phase equilibrium assays (Stanley *et al.*, 1983). This point is emphasized in Table 1 in which the affinity ratios of a panel of monoclonal anti-DNP antibodies deter-

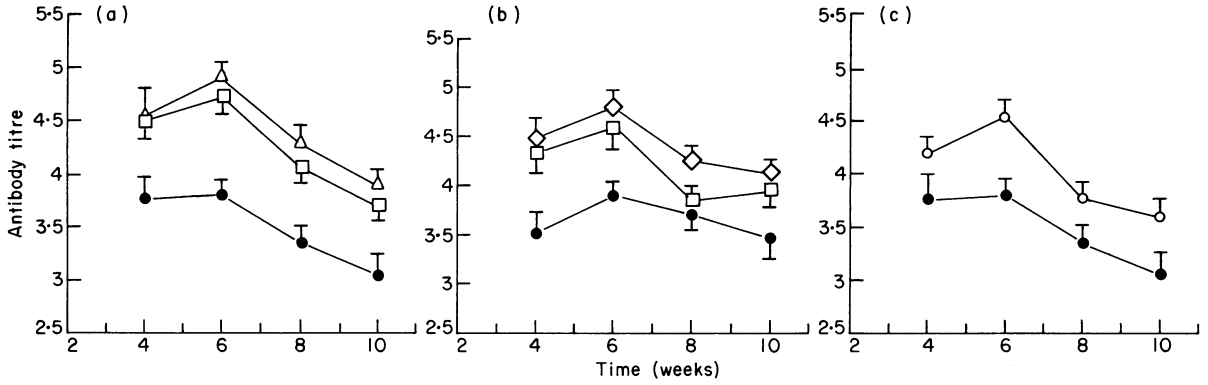

Fig. 1. The effect of interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β) peptide and tuftsin on the anti-DNP antibody titre assessed by ELISA in low affinity non-maturing (N/M) mice. (a) □, IFN-γ 10000 U ($P<0.001$); △, IFN-γ 30000 U ($P<0.001$); ●, lipopolysaccharide (LPS) control. (b) ◇, IL-1 peptide 100 mg/kg ($P<0.002$); □, IL-1 peptide 200 mg/kg; ($P<0.01$); ●, control peptide. (c) ○, tuftsin 25 μg ($P<0.005$); ●, LPS control. Values represent $\log_{10}$ titre + s.e.m. from groups of six mice. Significance was calculated using Students *t*-test for the difference at bleed 4 between test results and control LPS group.

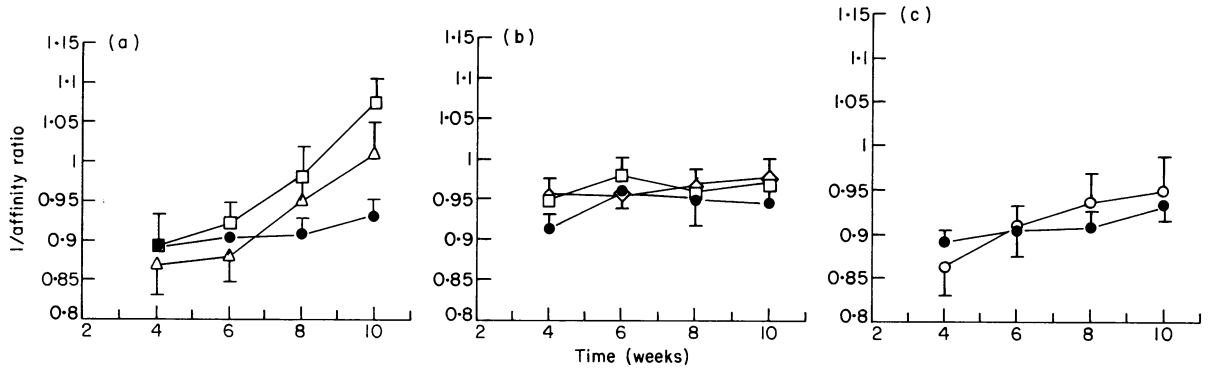

Fig. 2. The effect of interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β) peptide and tuftsin on the anti-DNP antibody affinity assessed by inhibition enzyme immunoassay in low affinity non-maturing (N/M) mice. (a) □, IFN-γ 10000 U ($P<0.01$); △, IFN-γ 30000 U; ●, lipopolysaccharide (LPS) control. (b) ◇, IL-1 peptide 100 mg/kg; □, IL-1 peptide 200 mg/kg; ●, control peptide. (c), tuftsin 25 μg; ○, LPS control. Values represent mean ± s.e.m. of the reciprocal of the ratio of the affinity of the test serum to that of the standard anti-DNP antibody from groups of six mice. Only in the group receiving 10000 U was the mean significantly different from the control.

Table 1. Comparison of anti-DNP affinity values of a panel of monoclonal antibodies assessed by three different methods

Monoclonal antibody	Equilibrium dialysis* ($\times 10^6$ M^{-1})	Globulin precipitation* ($\times 10^6$ M^{-1})	Inhibition EIA (1/affinity ratio)
49	41·3 ± 26·1	2·3 ± 1·6	1·62 ± 0·02
53	16·5 ± 1·7	1·1 ± 0·5	1·04 ± 0·02
51	11·1 ± 7·4	0·5 ± 0·2	0·96 ± 0·02
57	0·35 ± 0·02	0·6 ± 0·3	0·68 ± 0·03

Values represent means of replicate estimations ± s.d.
* Data from Stanley *et al.* (1983).
EIA, enzyme immunoassay.

mined by this assay are compared with affinity values obtained by the fluid-phase equilibrium dialysis and globulin precipitation assays. Thus a > 100-fold difference in affinities of these antibodies as measured by equilibrium dialysis and a 4·5-fold difference by globulin precipitation is represented by a 2·4-fold difference as assessed by the inhibition enzyme immunoassay.

Further evidence for the effectiveness of IFN-γ in increasing antibody affinity is given in Fig. 3, which represents histograms of antibody affinity sub-populations in groups of two representative mice from each treatment group. Only those mice receiving IFN-γ (Fig. 3 groups b,c) showed an increase in the high affinity sub-populations (solid bars) in bleed 4 compared with bleed 1.

The ability of IFN-γ to potentiate antibody affinity as assessed by the inhibition enzyme immunoassay was confirmed in a separate group of experiments in which low N/M mice received the DNP-BGG antigen together with 1000, 5000 or

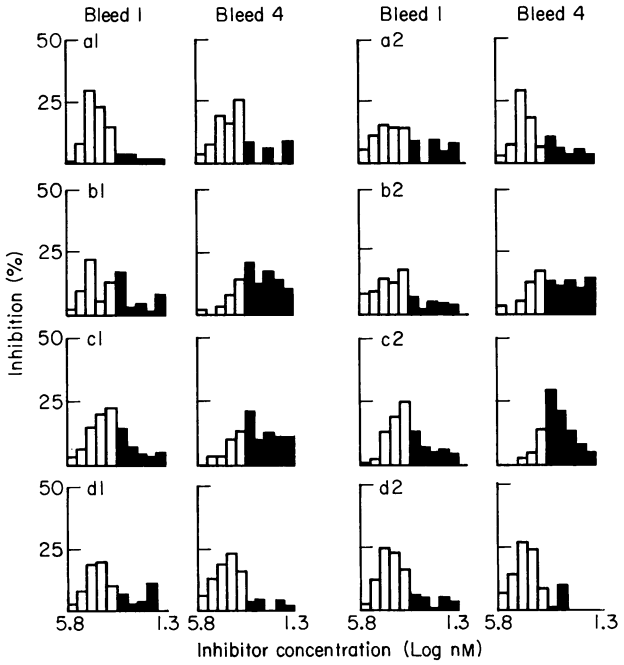

Fig. 3. The effect of interferon-gamma (IFN-γ) on the affinity of anti-DNP antibody affinity in low affinity non-maturing (N/M) mice. Histograms of the distribution of antibody affinity subpopulations in the sera of two representative mice from groups of animals receiving: a, lipopolysaccharide; b, 30 000 U IFN-γ; c, 10 000 U IFN-γ; and d, 25 μg tuftsin. Solid bars are for highlighting the shift in affinity subpopulation from low to high affinity.

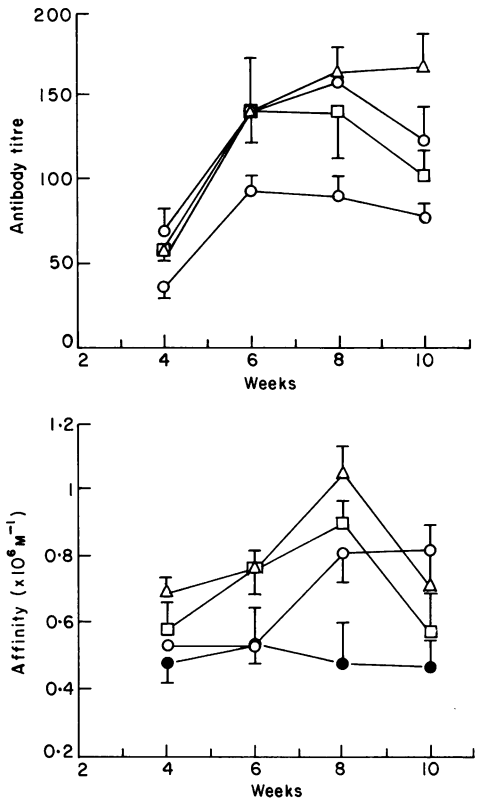

Fig. 4. The effect of interferon-gamma (IFN-γ) on the levels (Ab_t) and affinity (K, M^{-1}) of the anti-DNP antibody low affinity non-maturing (N/M) mice assessed by fluid-phase globulin precipitation assay. □, 1000 U IFN-γ; ○, 5000 U IFN-γ; △, 10 000 U IFN-γ; ●, controls receiving antigen only. Mean ± s.e.m. of groups of five mice. Significance was calculated using Student's t-test for the differences in the means in groups receiving IFN compared with controls and the results from bleed 3 were: Ab_t: 10 000 U, $P < 0{\cdot}01$; 5000 U, not significant; 1000 U, $P < 0{\cdot}05$. Affinity: 10 000 U, $P < 0{\cdot}001$; 5000 U, $P < 0{\cdot}05$; 1000 U, not significant.

10 000 U of IFN-γ and the affinity of antibody determined by the fluid-phase globulin precipitation assay. The results (Fig. 4) confirm the ability of the interferon to potentiate both antibody levels (expressed as Ab_t, pM of antigen bound by 10 μl serum) and affinity compared to mice injected with antigen in saline alone.

DISCUSSION

The work described here was performed in order to seek an immunopotentiating agent which could not only enhance antibody levels, but also the production of high affinity antibody following simultaneous administration with antigen. Three immunomodulating agents (IFN-γ, IL-1β nonapeptide and tuftsin) were assessed for their ability to potentiate antibody levels and affinity in low N/M mice following intraperitoneal administration with the antigen DNP-BGG. These mice were selected for this study because they have the characteristic of failing to show the normal progressive rise in antibody affinity with time (maturation) after immunization with antigen in FCA. All three agents enhanced antibody levels, but only IFN-γ was able to potentiate affinity maturation. One simple explanation for this effect of IFN-γ could be that it affected the immunoglobulin class or subclass distribution of the antibody response. While this was not addressed directly in these studies, other experiments with this cytokine in low N/M mice (M. E. Devey, personal communication) have demonstrated that in IFN-γ-treated animals, anti-DNP antibody levels, measured by an ELISA, were increased in all IgG subclasses with no particular subclass being preferentially affected. Whether the adjuvant affected the affinity of antibody in any particular class or subclass has not been investigated.

Previous work on the cellular basis of the failure of affinity maturation in low N/M mice has indicated a role for T suppression (Steward, Stanley & Furlong, 1986) and recent experiments employing *in vivo* T cell depletion techniques have shown that $CD8^+$ cells play an important role in preventing affinity maturation in these mice (Holland & Steward, 1989). Interestingly, affinity maturation could be demonstrated in low N/M mice following subcutaneous immunization and this suggests differences in antigen handling following intraperitoneal compared with that following subcutaneous immunization. Furthermore, the induction of MHC class II molecules is deficient in low N/M mice compared with high affinity mice, that are capable of normal affinity maturation (Phillips, 1988).

Of the three agents used in the present study, only IFN-γ is established as an inducer of MHC and the possibility arises therefore that the effect of IFN-γ on antibody affinity in these mice may be due to its ability to affect class II expression on peritoneal antigen-presenting cells. It has been proposed that the level of MHC class II expression may be involved in the

differential activation of T helper and T suppressor precursors. Using the GAT system (L-glutamic acid–L-alanine–L-tyrosine), Pierres & Germain (1978) demonstrated that both responder and non-responder mice could make T-suppressor cells specific for GAT and suggested that the difference between responders and non-responders related to the ability of their macrophages to present antigen efficiently. More recently, Sadegh-Nasseri, Dessi & Sercarz (1986) equated non-responsiveness to HEL to insufficient class II MHC expression. A reversal of H-2-linked unresponsiveness to lysozyme was demonstrated which is consistent with the expression of reversing non-H-2 genes resulting from the balance of antigen presentation to T helper and T suppressor cells in H-2^b mice. In this system, T suppressor cells play a dominant role and these authors suggested that inefficient processing of the antigen resulted in more effective triggering of these cells in the non-responder C57BL/6 and A.BY mice while the reverse may be the case in responder BALB/b mice. This view is supported by observations of Shastri *et al.* (1984) which demonstrate that C57BL/6 B cells and antigen presenting cells failed to cooperate with syngeneic T helper cell clones to produce antibody in the absence of added concanavalin A (Con A) supernatant. Con A supernatant contains several differentiation and stimulating factors, including IFN-γ, and has been shown to enhance class II MHC expression of macrophages (McNicholas, King & Jones, 1983). This work highlights the importance of the quantitative expression of the class II-processed antigen complex and is consistent with the proposal that variation in class II antigen expression could play a central role in immune regulation (Janeway *et al.*, 1984).

Since IFN-γ is known to enhance class II expression, it is possible that administration of IFN-γ to low N/M mice results in enhanced class II expression and stimulation of T helper cells and affinity maturation in preference to the stimulation of suppressor cells. If this interpretation is correct, then the failure of the IL-1β peptide and tuftsin to affect affinity maturation is not surprising, since there is no evidence to suggest that these agents affect class II antigen expression. The tetrapeptide tuftsin is reported to exert its activity by the stimulation of several functions of macrophages including phagocytosis, pinocytosis, antigen processing and motility (Tzehoval *et al.*, 1980). While the mechanisms by which IL-1 exerts its activity are not clear, it has been suggested that the adjuvant action of this cytokine could result from the induction of IL-2 and IL-2 receptor expression on T cells (Oppenheim, Stradler & Meltzer, 1978; Mizel & Ben-Zvi, 1980; Granstein *et al.*, 1985). The *in vivo* activity of IL-1 has been shown to be mimicked by a synthetic nonapeptide representing residues 163–171 (VQGEESNK) of the native polypeptide (Antoni *et al.*, 1986; Nencione *et al.*, 1987; Frasca *et al.*, 1988). It is of interest in this context that IL-1α, which lacks the VQGEESNK sequence, is devoid of *in vivo* immunostimulatory activity (Boraschi *et al.*, 1989). The observation that recombinant IL-1α fails to potentiate antibody responses in low N/M mice is consistent with this view (G.P. Holland, unpublished observations).

The results presented here suggest that IFN-γ has the important characteristic of potentiating antibody affinity and antibody levels. There are other adjuvants which are capable of enhancing antibody levels but the property of IFN-γ to also enhance affinity makes it an important potential adjuvant for vaccine use in humans since it is likely that in the outbred human population, genetically controlled differences in the affinity of the antibody response to certain antigens may well be expected.

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