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Symposium on 'The molecular and cellular roles of nutrients in the immune system'

All-*trans*-retinoic acid and polyriboinosinoic : polyribocytidylic acid cooperate to elevate anti-tetanus immunoglobulin G and immunoglobulin M responses in vitamin A-deficient Lewis rats and Balb/c mice

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Vitamin A (VA) deficiency compromises antibody responses to T-cell-dependent antigens such as tetanus toxoid, but this effect can be reversed through administration of retinol or retinoic acid (RA). To test whether RA and polyriboinosinioc : polyribocytidylic acid (PIC), a known inducer of several forms of interferon (IFN), can cooperate to increase specific immunoglobulin (Ig)G and IgM production during VA deficiency, rats and mice were made VA-deficient, immunized with TT and treated with all-*trans*-RA, PIC or their combination. VA-deficient rats produced low primary and secondary anti-tetanus IgG responses (VA-deficient controls *v*. VA-sufficient controls $P<0{\cdot}001$), although total IgG was slightly elevated when compared with VA-sufficient control rats. Although RA administered alone elevated antibody production during VA deficiency to control levels, RA combined with PIC synergistically enhanced these responses (RA and PIC group *v*. all other groups $P<0{\cdot}0001$). In contrast, Balb/c mice maintained on a VA-deficient diet and immunized in a similar fashion showed no impairment in antigen-specific IgG levels, but treatment with a combination of RA and PIC still evoked an additive enhancement in antigen-specific antibody production. Additionally, RA and PIC administration to VA-sufficient mice resulted in elevated antibody responses, suggesting that this combination should be evaluated further for its immuno-stimulatory effects.

Vitamin A: Retinoic acid: Polyriboinosinic acid : polyribocytidylic acid: Interferon: Antibodies

Vitamin A (VA) is required for many biological processes including reproduction, growth, vision and proper functioning of the immune system (for review, see Ross, 2000*a*). In its absence widespread alterations develop in immune system cellularity and immunological processes, leading to impairment or dysregulation of functional responses (Ross, 1992). Numerous processes that are important to host defence have been shown to be altered in VA-deficient animals and human subjects, including impaired T- and B-cell functions, decreased natural killer cell activity, atrophy or sometimes enlargement of immune tissues and impaired antibody responses to protein and polysaccharide antigens (for review, see Ross, 1992; Ross & Hämmerling, 1994). VA deficiency has long been associated with increased risk or severity of infectious diseases in animals and human subjects. An increased severity of measles, malaria, human immunodeficiency virus, diarrhoeal diseases and some respiratory infections in VA-deficient populations has received significant attention, and is thought to be a significant cause of young child mortality in parts of the developing world where VA deficiency is still prevalent (Semba, 1994). The efficacy of VA in reducing morbidity and mortality has led to the introduction of VA supplementation programmes in many affected regions (Beaton *et al*. 1993; Fawzi *et al*. 1993; Glasziou & Mackerras, 1993), and to new epidemiological

Abbreviations: IFN, interferon; Ig, immunoglobulin; IRF, interferon regulator factor; PIC, polyriboinosinoic : polyribocytidylic acid; RA, retinoic acid; STAT, signal transducer and activator of transcription; TT, tetanus toxoid; VA, vitamin A.
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and clinical studies to further probe the ability of VA to improve public health (Sommer & West, 1996). Various mechanisms have been proposed as causes of VA deficiency including, besides inadequate intake of the vitamin, an increased rate of utilization and accelerated urinary losses of VA (see Semba, 1994).

Tetanus infection constitutes a very significant public health problem in much of the developing world, estimated to affect approximately 800 000 neonates and young children per year (Henderson *et al.* 1988). To better understand the relationship between VA status and resistance to infection, our laboratory has developed a model of tetanus toxoid (TT) immunization in experimental animals to investigate the effect of VA deficiency on antibody production. TT is a potent T-cell-dependent antigen that stimulates T-cells to clonally proliferate and B-cells to secrete antibodies of the immunoglobulin (Ig)M and IgG classes. In studies previously reported and reviewed (Pasatiempo *et al.* 1990; Kinoshita *et al.* 1991; Kinoshita & Ross, 1993; Ross, 1996, 2000*b*), we showed that VA deficiency in a Lewis rat model compromises the production of both primary and secondary TT-specific IgM and IgG responses. In the primary antibody response this reduction in anti-TT IgG was due primarily to a decrease in the rat IgG2b subclass, while during the secondary response, all subclasses were low (Kinoshita & Ross, 1993). Following provision of retinol (Kinoshita *et al.* 1991; Kinoshita & Ross, 1993) or RA (DeCicco *et al.* 2000), these responses were restored to values similar to those of control rats, suggesting that memory cell formation during the primary response is not affected by VA deficiency. Similarly, such cells, once formed, can be reactivated by TT to elicit memory effector cells which produce a quantitatively and kinetically normal secondary anti-TT IgG response.

Retinoic acid (RA) is the most potent known active metabolite of retinol, and its immuno-potentiating properties on lymphoid cells have been studied in normal intact mice and rats and in isolated cells (Ross & Hämmerling, 1994; Ross, 2000*b*). These properties include enhanced thymocyte activation to mitogens (Abb & Deinhardt, 1980; Valone & Payan, 1985; Dillehay *et al.* 1987) and augmented antibody production from B-cells (Wang & Ballow, 1993). Two isomers of RA function in a hormonal capacity as the ligand for two classes of nuclear receptors, the RAR or RXR, which belong to the steroid receptor superfamily (for review, see Mangelsdorf *et al.* 1995). Once RA has bound to the receptor ligand-binding domain, the heterodimer RAR/RXR binds to RA response elements located in gene promoters, leading to transcriptional activation, or sometimes repression, of specific target genes. Despite considerable understanding of RA-regulated gene expression *in vivo* and in cells, the physiological regulation of gene expression by VA and RA in intact organisms is still poorly understood.

Interferons (IFN) are a family of cytokines whose production is elicited in response to stimuli such as foreign cells, bacteria and viral antigens (Sen & Ransohoff, 1993). Polyriboinosinoic : polyribocytidylic acid (PIC), a synthetic double-stranded polyribonucleotide, mimics double-stranded RNA viruses and has long been known as a strong inducer of IFN (Field *et al.* 1967; Hilleman, 1970). The anti-infective activity of PIC has been demonstrated in several models of viral disease and malaria (Coppenhaver *et al.* 1995; Wong *et al.* 1995; Puri *et al.* 1996). PIC elicits production of large amounts of IFN-α and interleukin 12 (Manetti *et al.* 1995), both of which prime lymphocytes to produce IFN-γ (Manetti *et al.* 1994), ultimately boosting cell-mediated immunity. Additionally, PIC stimulates humoral immunity and enhances B-cell activation, and up regulation of surface IgG receptors on monocytes has been reported (Schmidt & Douglas, 1976).

The two major classes of IFN produced by PIC, type I (IFN-α and -β) and type II (IFN-γ), signal via distinct plasma membrane receptors which utilize similar but non-identical signal transduction pathways (for review, see Williams & Haque, 1997). Both pathways involve the phosphorylation of members of the Janus protein tyrosine kinase family, followed by phosphorylation of two members of the signal transducers and activator of transcription (STAT) family of latent cytoplasmic factors. The phosphorylated STAT proteins, on activation, migrate to the nucleus and bind as heterodimers or homodimers to the promoter regions of IFN-responsive genes. Although there are many different members of the STAT family, STAT-1 is recruited by the binding of both type I and type II IFN to their respective receptors, and therefore serves as a potential link between type 1 and type II IFN signalling pathways (Darnell *et al.* 1994; Williams & Haque, 1997).

It has been reported that in cultured cells, all-*trans*-RA induced STAT-1 expression via RA receptor binding to an RA response element located in the 5′-regulatory region of the STAT-1 gene (Darnell *et al.* 1994; Matikainen *et al.* 1997; Weihua *et al.* 1997). This increased level of STAT 1 could amplify the signal transduction pathway, and ultimately the expression of target genes. Other genes induced by IFN include members of the interferon regulator factor (IRF) family. Two of these members, i.e. IRF-1 and IRF-2, regulate IFN-inducible gene expression and cellular proliferation through induction of target genes (Harada *et al.* 1989). These two factors act in opposing manners, with IRF-1 functioning as a transcriptional activator, and IRF-2 as a transcriptional repressor. Both the mRNA and protein levels of IRF-1 and IRF-2 were increased by addition of RA to leukaemia cells *in vitro* (Pelicano *et al.* 1997; Matikainen *et al.* 1998).

The anti-proliferative effects of retinoids and IFN administered individually are well known, but their utility as single agents in treating cancers and infections is often limited by significant side effects. However, a growing body of evidence now supports the potential for using these two agents in combination, at lower doses. The combination of RA and IFN, especially type I IFN, has resulted in additive or synergistic effects in cells and in anti-tumour models *in vivo* (for review, see Bollag *et al.* 1994; Moore *et al.* 1994). When administered together retinoids and IFN have synergistically induced cell differentiation, while inhibiting tumour growth, size and angiogenesis (Wuarin *et al.* 1991; Majewski *et al.* 1994; Lippman *et al.* 1997). Although the molecular mechanisms are not fully elucidated, we (DeCicco *et al.* 1999, 2000) and other researchers (Matikainen *et al.* 1997, 1999) have suggested possible genes shared in the signalling pathways of both IFN

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and retinoids that may explain how these two agents cooperate.

To our knowledge, no one has tested the combination of RA and PIC in an *in vivo* model of immune function. The present article focuses on antibody production, first reviewing data from a recent study in rats which was designed to determine whether the combination of RA and PIC could cooperate to enhance antibody responses during VA deficiency (DeCicco *et al*. 2000). We found that the combination of RA and PIC synergized to evoke a heightened antigen-specific antibody response in the VA-deficient Lewis rat. We also present new data from similar studies of the antibody response of Balb/c mice. In this model RA and PIC acted additively to elevate anti-TT specific antibody responses in mice fed on control and VA-free diets. Differences between these models are discussed and an hypothesis for the interaction of RA and PIC is presented.

Materials and methods

Experimental design for rat experiments

Animal procedures were approved by the Institutional Animal Use and Care Committee of The Pennsylvania State University. Details of the rat model and immunization procedures can be found in other publications (Kinoshita *et al*. 1991; DeCicco *et al*. 2000). Briefly, in this study three lactating dams were fed on a semi-purified nutritionally-adequate diet (AIN-93G; Reeves *et al*. 1993), modified to contain no VA, from the time of receipt. At 21 d of age thirty male Lewis rats were randomized into five groups and fed on either the complete AIN-93G diet containing 4 mg retinol equivalents/kg diet (VA-sufficient group; *n* 6) or continued on the VA-deficient diet (*n* 24). The food intake of VA-sufficient rats was controlled by pair-feeding to equalize the body weights of the VA-sufficient and VA-deficient rats. By 40 d of age, rats fed on the VA-deficient diet had serum retinol concentrations, as determined by reverse-phase HPLC (Ross, 1990) below 0·2 µmol/l (*v*. >1 µmol/l for controls) and were considered to be VA deficient.

The treatment and schedule protocols for rat studies are illustrated in Tables 1 and 2 respectively. Groups of six rats per treatment received one of the five treatments, and all rats were immunized intraperitoneally with 100 µg TT (Connaught Laboratories, Ontario, Canada) in 1 ml saline (9 g NaCl/l) when 41 d old. Groups treated with RA received 100 µg all-*trans*-RA (Sigma, St Louis, MO, USA) solubilized in 20 µl rapeseed oil orally every 48 h for 1 week beginning 1 d before immunization. Groups treated with PIC, in the form of PIC-poly-L-lysine carboxymethylcellulose, received 20 µg in 0·5 ml saline intraperitoneally on the day of immunization. Rats receiving RA alone or in combination with PIC began to gain weight at a rate comparable with that of the control animals after receiving treatments (data not shown), at which time pair-feeding was continued only in VA-deficient rats receiving vehicle only and rats treated only with PIC. At the times indicated in Table 2, all animals were bled via the tail vein for serum antibody determinations. At 3 weeks after primary immunization all rats were re-immunized with TT, as described earlier, but they received no other treatments. At 7 d after the second immunization all rats were bled from the tail vein and serum was prepared and frozen at –20° for determination of serum anti-tetanus IgG and IgM, and total IgG.

Table 1. Diet and treatment groups for Lewis rats

Group (*n* 6)	Diet	Treatment
		(All-*trans*-retinoic acid (RA), polyriboinosinic acid : polyribocytidylic acid (PIC)*, or vehicle† and saline (9 g NaCl/l) control)
1	Vitamin A (VA)-deficient	Vehicle and saline control
2	VA-deficient	RA and saline
3	VA-deficient	Vehicle and PIC
4	VA-deficient	RA and PIC
5	VA-sufficient	Vehicle and saline control (pair-fed)

* Stabilized with poly-L-lysine and carboxymethylcellulose.
†Vehicle for RA was rapeseed oil.

Experimental design for mouse experiments

Eighteen virgin female and six male Balb/c mice were purchased from Charles River Laboratories (Kingston, NY, USA) when they were 7 weeks old. All the mice were fed on the same semi-purified vitamin A-free diet (AIN-93G) as in the rat study, beginning 1 week before breeding was initiated. Mice were continuously fed this diet during breeding, pregnancy and lactation. After weaning the pups remained on the VA-free diet until they were bred at 7 weeks of age. The second-generation mice that resulted from this breeding therefore were the offspring of parents that had never received VA in their lifetime. Unlike the easily VA-depleted rats, mice are highly efficient at conserving VA and therefore require more time to become deficient (Smith *et al*. 1987; Smith, 1990). The second-generation pups were weaned when 21 d old, after which time they were fed on either control AIN-93G diet containing 4 mg retinol equivalents/kg diet or continued on

Table 2. Treatment schedule for studies in rats and mice*

Day –1	Pre-immunization blood withdrawal
Day 0	100 µg All-*trans*-retinoic acid (RA) or vehicle†; 20 µg (rats) or 2 µg (mice), polyriboinosinic acid : polyribocytidylic acid (PIC)‡, or saline (9 g NaCl/l); Immunization with 100 µg (rats) or 10 µg (mice) tetanus toxoid (TT)
Day 3,5	100 µg RA or vehicle orally
Day 6,10	Collect blood for primary immunoglobulin responses
Day 20	Pre-secondary immunization blood collection
Day 21	Secondary immunization with 100 µg (rats) or 10 µg (mice) TT
Day 28	Collect blood for total IgG, secondary anti-TT IgG, and isotype-specific IgG analysis (rats)

* Treatment day 0 corresponds to 41 d of age in rats, when the serum retinol concentration of VA-deficient rats was <0·2 µM. Treatments were begun when mice were 16 weeks old and the serum retinol concentration of VA-deficient mice was < 0·1 µmol/l.
† Rapeseed oil.
‡ Stabilized with poly-L-lysine and carboxymethylcellulose.

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the VA-deficient diet. Pups were weighed weekly and when the weight of the mice fed on the VA-deficient diet was significantly less than that of the control mice, pair-feeding was initiated (9 weeks of age for males and 11 weeks of age for females).

Seventy-two second-generation mice resulted from this breeding. Males and females were equally divided into different treatments (nine mice per group). Previously-conducted experiments had confirmed that the anti-TT antibody response does not differ between the sexes (KL DeCicco, unpublished data, not shown). The treatments given to the mice are depicted in Table 3, and the immunization schedule is the same as that for the rat experiments described earlier, as shown in Table 2. Note that to address whether RA and PIC had effects on normal animals, four additional treatment groups using the same treatments in mice fed on the VA-sufficient control diet were included in this experiment.

ELISA procedures

Assays for TT-specific IgM and IgG were performed essentially as described previously (Kinoshita *et al*. 1991; DeCicco *et al*. 2000). Serial dilutions of plasma were performed on serum samples to assure reactions were in the linear range of detection. Additionally, a pooled serum standard was prepared from TT-immunized rats or mice and run on each ELISA plate. All data points were plotted and calculated in units based on a standard curve, where one unit was defined as the response of a 1/10 000 dilution of standard serum for IgG and a 1/200 dilution for IgM.

Statistical analysis

Anti-TT IgG and IgM responses were log-transformed (base 10) before statistical analysis, and geometric means are presented. Unpaired *t* tests were conducted on VA-deficient *v*. VA-sufficient groups to assess the effect of nutritional status. $P<0{\cdot}05$ was considered significant. The effects of RA and PIC in VA-deficient rats and VA-deficient or VA-sufficient mice were evaluated using two-way ANOVA in a Latin-square design. Main effects were further analysed using a Tukey-Kramer *post hoc* test (SuperAnova; Abacus Concepts, Berkeley CA, USA).

Table 3. Diet and treatment groups for Balb/c mice

Group (*n* 9)	Diet	Treatment
		(All-*trans*-retinoic acid (RA), polyriboinosinic acid : polyribocytidylic acid (PIC)*, or vehicle† and saline (9 g NaCl/l) control)
1	Vitamin A (VA)-deficient	Vehicle and saline control
2	VA-deficient	RA and saline
3	VA-deficient	Vehicle and PIC
4	VA-deficient	RA and PIC
5	VA-sufficient	Vehicle and saline control (pair-fed)
6	VA-sufficient	RA and saline
7	VA-sufficient	Vehicle and PIC
8	VA-sufficient	RA and PIC

* Stabilized with poly-L-lysine and carboxymethylcellulose.
† Vehicle for RA was rapeseed oil.

Results

Antibody responses to tetanus toxoid immunization in vitamin A-deficient Lewis rats are increased synergistically by retinoic acid and polyriboinosinic acid: polyribocytidylic acid

The primary anti-tetanus IgM was measured 6 d after immunization, as this time point has been previously determined to be the peak of the plasma anti-TT response. Although anti-tetanus IgM was slightly lower in the VA-deficient group, this difference did not reach statistical significance. However, RA reversed this effect, as anti-tetanus IgM was approximately the same in the VA-deficient RA-treated group and the VA-sufficient control group (Fig. 1(A)). PIC had a significant effect ($P<0{\cdot}003$) on anti-TT IgM production, elevating IgM levels 4-fold in comparison with the VA-sufficient control group (11·82 *v*. 3·2 units IgM/ml). The combination of RA and PIC evoked a synergistic response which was approximately thirty times greater than that of VA-deficient rats (56·5 *v*. 1·9 units

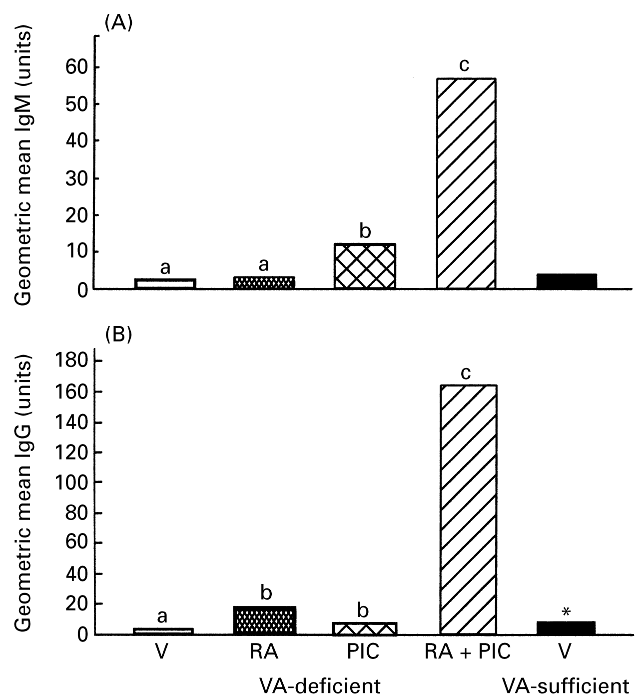

Fig. 1. Anti-tetanus immunoglobulin (Ig) M (A) and IgG (B) primary antibody responses in Lewis rats measured 7 and 10 d respectively after immunization with tetanus toxoid. Data shown are the geometric means for six rats per group. For details of diets and procedures, see pp. 521–522. a, b, c Vitamin A (VA)-deficient groups with different superscript letters were significantly different ($P<0{\cdot}015$ for IgM, $P<0{\cdot}0001$ for IgG; least-squares means test). The value for IgG for the pair-fed control group was significantly different from that for the the VA-deficient group: *$P<0{\cdot}03$ (*t* test). V, vehicle only (▭, ▬); RA, retinoic acid (▦); PIC, polyriboinosinic : polyribocytidylic acid (▩); VA-sufficient, VA-sufficient control group (▬); (▨), RA + PIC. (Modified from DeCicco *et al*. 2000, with the permission of University of Chicago Press.)

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IgM/ml) and seventeen times greater than that of pair-fed controls (56·5 *v.* 3·2 units IgM/ml).

The primary anti-tetanus IgG response was measured 10 d after immunization, the previously-determined peak of the anti-TT IgG response (Kinoshita *et al.* 1991). When compared with VA-sufficient pair-fed control rats, VA-deficient rats produced a very weak TT-specific primary IgG (7·9 *v.* 2·0 units IgG/ml) response, as shown in Fig. 1(B). Provision of RA alone increased the antigen-specific anti-TT IgG response (16·6 units IgG/ml) to a level greater than that of the control group. PIC alone also elevated the anti-tetanus IgG response (5·3 units IgG/ml), although not as high as control values. The combination of RA and PIC synergized to evoke a response approximately twenty times greater than that of the control group (162·2 *v.* 7·9 units IgG/ml). By a two-way ANOVA of log (base 10) transformed data, there were main effects of RA and PIC ($P<0·0001$), as well as an interaction ($P=0·025$). The difference in response between VA-deficient and VA-sufficient untreated rats was also highly significant ($P<0·001$; *t* test).

All rats were re-immunized with TT 21 d after the first immunization, but they received no additional treatments at this time. Blood was collected 7 d after re-immunization for total and antigen-specific IgG, as well as for isotype-specific IgG analysis. As shown in Fig. 2, VA-deficient animals produce a significantly ($P<0·0001$) weaker TT-specific secondary IgG response (18·6 units IgG/ml) when compared with control rats (219 units IgG/ml). Although no further treatments were administered along with the second TT immunization, the secondary anti-TT IgG response of the RA-treated VA-deficient rats was still elevated 19-fold. Since RA is quickly metabolized (el Mansouri *et al.* 1995), no RA is expected to have been present at this time. TT-specific IgG response in rats treated with PIC alone (30·9 units IgG/ml) did not differ from that of untreated VA-deficient rats. However, the group treated with the combination of RA and PIC showed a greatly synergistic response, which was approximately fifty-four times greater (11 749 *v.* 219 units IgG/ml) than that of VA-sufficient control rats and 632 times greater (11 749 *v.* 18·6 units IgG/ml) than that of VA-deficient rats. The interaction between RA and PIC was also highly significant ($P<0·0001$; two-way ANOVA). An analysis of anti-TT IgG isotypes was also conducted on serum samples taken after primary and secondary immunization. Values for VA-deficient rats were low for all isotypes of antigen-specific IgG (data not shown). RA administration greatly elevated IgG1 and IgG2a levels, whereas PIC primarily induced IgG2b. All isotypes were elevated in rats treated with the combination of RA and PIC.

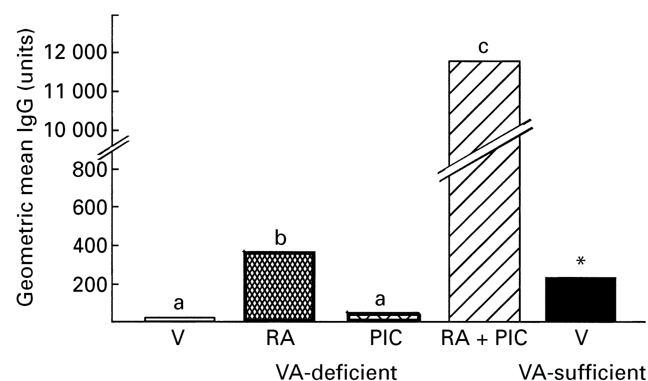

Fig. 2. Anti-tetanus immunoglobulin (Ig)G secondary response 7 d after re-immunization with tetanus toxoid in vitamin A (VA)-deficient rats previously treated with retinoic acid (RA; ▬)- or polyriboinosinic : polyribocytidylic acid (PIC; ▒) or their combination (▨) at the time of the primary immunization. Data shown are the geometric means for six rats per group. For details of diets and procedures, see pp. 521–522. a, b, c VA-deficient treatment groups with different superscript letters were statistically different ($P<0·0001$; least-squares means test). The value for the pair-fed control group (VA-sufficient group) was significantly different from that for the VA-deficient group: $^{*}P=0·008$. V, vehicle only (▭, ▬). (From DeCicco *et al.* 2000, with the permission of University of Chicago Press.)

Total IgG concentrations in individual rats were measured on the same plasma samples used to assay the secondary anti-tetanus IgG response. Group means were (mg/ml) 2·1, 2·5, 2·7, 3·2 and 1·5 for VA-deficient, RA-treated, PIC-treated, RA and PIC-treated, and VA-sufficient groups respectively. Although some of these differences were statistically significant (PIC, and PIC and RA *v.* other groups $P<0·05$; DeCicco *et al.* 2000), the effect of RA and PIC on total IgG was small in relationship to the strong effect that this combined treatment had on specific IgG production elicited by TT immunization.

Antibody responses in Balb/c mice: additive enhancement by retinoic acid and polyriboinosinoic acid: polyribocytidylic acid

As shown in Table 2, the primary IgG response of TT-immunized mice was measured 10 d after immunization. Although the serum retinol levels in the mice fed on the VA-free diet were almost undetectable, the mice maintained approximately normal weights and their immune functions were also approximately normal. (The body weights of VA-deficient male and female mice at the end of the study were within 1 and 0·5 g respectively of those of VA-sufficient control mice. The serum retinol levels of VA-deficient mice were undetectable (< 0·005 µM) early in the depletion phase, but mice remained active. A few VA-deficient mice died without showing evident weakness beforehand. Thus, VA deficiency in mice differed noticeably from the classical form observed in rats in which significant weight loss precedes morbidity and death (Dowling & Wald, 1960).) Unlike in the rat experiment, the primary anti-TT IgG levels of these mice were not greatly impaired (78 *v.* 145 units IgG/ml; Fig. 3(A)). We found that treatment with either RA or PIC individually increased the antigen-specific antibody response 5-fold (355 and 363 units IgG/ml respectively), to levels greater than those of the controls. The response to treatment with the combination of RA and PIC (832 units IgG/ml) was additive rather than synergistic in this mouse model. This response was still eleven times greater than that of the VA-deficient control mice and six times greater than that of the VA-sufficient control mice. By a two-way ANOVA of log (base 10)-transformed data, there were main effects of RA and PIC ($P<0·0001$).

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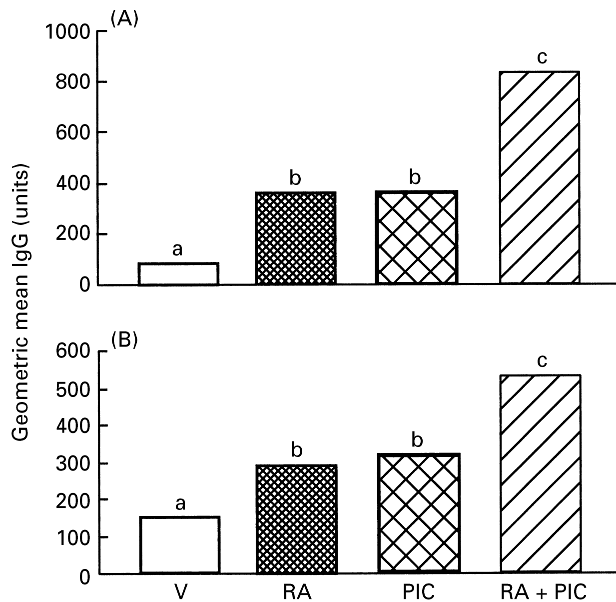

Fig. 1. Anti-tetanus IgG primary response in vitamin A (VA)-deficient (A) or vitamin A-sufficient Balb/C mice (B) treated with retinoic acid (RA; ▩), polyriboinosinic : polyribocytidylic acid (PIC; ▩), or their combination (▨) and immunized with tetanus toxoid. For details of diets and procedures, see pp. 521–522. Data shown are the geometric means for nine mice per group. VA-deficient treatment groups with different superscript letters were significantly different ($P<0{\cdot}0001$ for VA-deficient mice and $P<0{\cdot}02$ for VA-sufficient (control) mice respectively; least-squares means test). V, vehicle only (▭).

When mice of normal VA status were treated in an identical manner with RA, PIC or their combination, administration of RA and PIC individually resulted in mean anti-TT IgG levels of 288 and 316 units IgG/ml respectively ($P=0{\cdot}02$ in each case when compared with untreated controls), and elevated primary anti-tetanus IgG response 2-fold (Fig. 3(B)). The combination of RA and PIC elevated the anti-TT IgG response to 525 units IgG/ml, an increase of approximately 4-fold when compared with the response of normal mice ($P<0{\cdot}0001$).

All mice were re-immunized with TT 21 d after the first immunization, but received no additional treatments at this time. Blood was collected 7 d after re-immunization for total and antigen-specific IgG analysis. There were no significant differences in secondary anti-TT antibody production in VA-deficient untreated controls when compared with VA-sufficient untreated controls (1445 *v.* 1142 units IgG/ml), as shown in Fig. 4. However, treating VA-deficient mice with RA or PIC significantly ($P<0{\cdot}0005$) elevated anti-TT IgG responses more than 3-fold (Fig. 4(A)). The combination of RA and PIC (9772 units IgG/ml) acted in an additive fashion to elevate anti-TT IgG levels almost 7-fold when compared with VA-deficient untreated mice, yielding a highly significant result ($P<0{\cdot}0001$).

In the study of VA-sufficient mice, treatment with RA (4074 units IgG/ml) or PIC (3467 units IgG/ml) also elevated anti-TT IgG levels more than 3-fold ($P<0{\cdot}0002$), as shown in Fig. 4(B). The combination of RA and PIC (5129 units IgG/ml) elevated anti-TT levels approximately 6-fold when compared with untreated control mice ($P<0{\cdot}0001$).

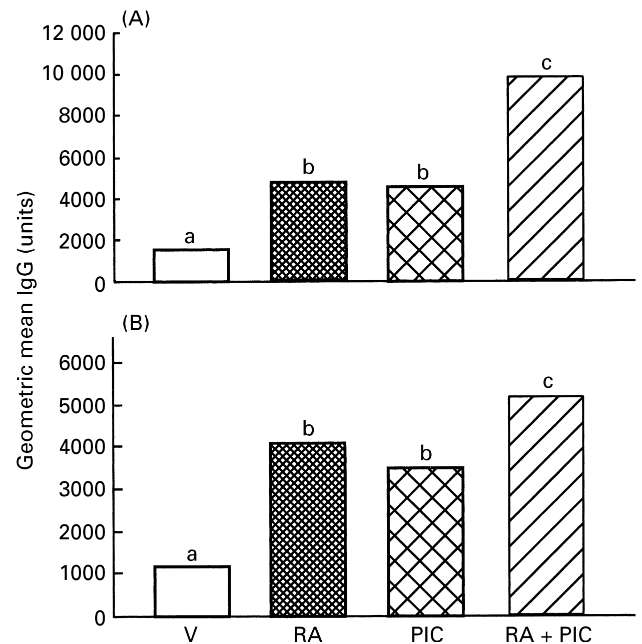

Fig. 4. Anti-tetanus IgG secondary response 7 d after re-immunization with tetanus toxoid in mice previously treated with retinoic acid (RA; ▩) or polyriboinosinic : polyribocytidylic acid (PIC; ▩) or their combination (▨) at the time of the primary immunization. (A) Vitamin A (VA)-deficient and (B) normal Balb/C mice. Data shown are the geometric means for nine mice per group. For details of diets and procedures, see pp. 521–522. VA-deficient treatment groups (A) with different superscript letters and VA-sufficient (control) treatment groups (B) with different superscript letters were significantly different ($P<0{\cdot}0005$ and $P<0{\cdot}0002$ respectively; least-squares means test). V, vehicle only (▭).

Total IgG concentrations from individual mice were measured on plasma samples used to assay the secondary anti-tetanus IgG response. They were found to be significantly ($P=0{\cdot}0002$) elevated in the VA-deficient mice (5·1 µg/ml) when compared with the VA-sufficient (1·3 µg/ml) controls, consistent with the hypergammaglobulinaemia previously reported for VA-deficient mice (Gershwin *et al.* 1984). Thus, although the TT-specific IgG responses were not lower in VA-deficient mice, their abnormally elevated total IgG levels suggested VA deficiency. RA or PIC administered alone did not significantly increase total IgG production, but the combination of RA and PIC significantly ($P<0{\cdot}0001$) increased total IgG concentration. Similarly in VA-sufficient mice treated with RA and PIC, the combination of RA and PIC stimulated total IgG production ($P<0{\cdot}008$ when compared with values for VA-deficient and VA-sufficient untreated mice).

Discussion

VA deficiency remains a significant public health problem, and a relationship between VA deficiency and infectious disease has been demonstrated in human clinical and epidemiological studies. Animal models provide a means to

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conduct carefully controlled experiments, in the absence of other nutrient deficiencies other than VA, to test for causality between VA status and immune function. Based on the congruence of human and animal studies that VA deficiency is a signficant risk factor for increased morbidity and mortality due to infections such as measles, malaria and tetanus, VA supplementation has been introduced in many developing countries as a public health intervention to reduce overall infection rate and mortality (Sommer & West, 1996).

PIC, a synthetic double-stranded RNA that mimics the effects of RNA viruses, has been shown to have generally beneficial effects on both cell-mediated and humoral immunity. Its principal mechanism of action is thought to be its ability to induce multiple forms of IFN, mainly IFN-α, although the full range of cytokines induced by PIC is not certain. Recently, there has been increased interest in testing the combination of RA and IFN for their anti-cancer activity, based on studies in cells which have shown synergistic interactions with respect to inhibition of cell growth (Wuarin *et al.* 1991), tumourigenicity (Lippman *et al.* 1997) and angiogenesis (Majewski *et al.* 1994). PIC is well tolerated (Hilleman, 1970), and has undergone limited testing in human clinical trials as well as more extensive study in animal models. Individually, each of these agents has limited anti-tumoural potential, in part due to the side effects associated with therapeutic doses, but as other researchers have discussed (Lippman *et al.* 1993; Moore *et al.* 1994), when administered simultaneously they often synergize due to overlapping properties. However, the combination of RA with PIC for possible cooperativity in an *in vivo* model of immune function, and specifically of antibody production, had not been tested. Based on the results mentioned earlier, we reasoned that these two agents may synergize to boost immunity, and thus we have tested whether RA and PIC can cooperate to boost antibody responses, even when animals were in an immuno-compromised state due to VA deficiency.

VA deficiency caused poor primary and secondary anti-tetanus responses, confirming previous reports (Pasatiempo *et al.* 1990; Kinoshita *et al.* 1991). Treatment with RA alone, provided to VA-deficient rats only with the primary immunization, significantly increased both the primary and secondary anti-tetanus IgG responses. This finding confirmed previous reports that RA has immuno-potentiating properties on lymphoid cells, including enhanced thymocyte activation to mitogens and augmented antibody production from B-cells. How RA enhanced this antibody production is not fully understood. Some investigators have reported that all-*trans*-RA can directly affect both B- and T-cells (Valone & Payan, 1985; Sidell *et al.* 1993; Ballow *et al.* 1996). In the case of T-cells it is likely that the direct effect occurs via the secretion of soluble cytokines that then effect B-cell growth or immunoglobulin secretion. We and other researchers have reported that on addition of RA there is an increase in mRNA expression for certain receptors for soluble cytokines involved in T-cell responses, such as the interleukin 2 receptor α (Sidell *et al.* 1993; Bhatti & Sidell, 1994) and β chains (Sidell *et al.* 1993; DeCicco *et al.* 2000).

An important finding in our rat study is that the secondary anti-tetanus response of RA-treated VA-deficient rats was of normal magnitude. The half-life of all-*trans*-RA in rats has been reported to be less than 2 h (el Mansouri *et al.* 1995); therefore the doses of RA provided at the time of primary immunization would have been entirely cleared long before re-immunization. The strong secondary anti-TT IgG response of previously-RA-treated rats implies that events at the time of primary immunization must have promoted the differentiation of new cell populations, or stimulated existing cells, which persisted and were capable of responding on antigen re-stimulation to provide antigen-specific memory. Such an effect would be consistent with the well-studied ability of RA to induce cell differentiation in a variety of cell models (Gudas *et al.* 1994). Currently, we are investigating whether certain cellular populations involved in T-cell-dependent antibody responses are expanded or shifted on addition of RA *in vivo*.

In contrast to the effects of RA on both primary and secondary responses, PIC stimulated only the primary anti-TT response in VA-deficient rats, suggesting that the cytokines elicited by PIC acted to amplify ongoing processes, but did not result in the generation of long-lived cell populations that became memory effector cells. This suggestion is consistent with the role of IFN-γ, one of the cytokines induced by PIC, acting to amplify cell responses, but not being by itself a strong growth factor. In mice, however, PIC treatment did result in a heightened secondary response, suggesting that the cytokines elicited in mice and rats, or their signalling pathways, might be somewhat different. IFN-α, which is likely to be the major form of IFN elicited by PIC treatment, may also stimulate ongoing processes but not function as strong growth and differentiation factors. As will be discussed, recent studies have pointed to type I IFN (IFN-α, IFN-β) as being important for T- and B-cell survival, and as preventing activation-induced T- and B-cell apoptosis in culture (Marrack *et al.* 1999; Mitchell *et al.* 1999; Su & David, 1999). In our studies in intact rats and mice the combination of RA and PIC in both species dramatically increased both the primary and secondary anti-tetanus IgG responses (Figs. 1 and 2). In the secondary response rats treated previously with RA and PIC produced anti-tetanus IgG levels that were dramatically elevated, being over 600-fold higher than those of VA-deficient untreated rats and 50-fold higher than those of VA-sufficient untreated rats. Thus, the synergistic stimulation by RA and PIC at the time of priming resulted in a markedly heightened memory response. This response occurred even as the VA deficiency of the rats had become severe, with impaired growth and anorexia evident in some of the animals by the end of the study. The synergism observed between RA and PIC suggests that both cell differentiation, likely to have been driven by RA, and enhancement of cellular responses, probably due to IFN, acted cooperatively to promote both primary antibody production and the generation of memory cells. We speculate that the memory cells induced in the primary response were capable of reacting at the time of the second immunization, without further treatment with RA or PIC, to become memory effector cells capable of high-output production of anti-tetanus IgG. In general, treatments had a

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similar effect on anti-TT IgM to that on IgG, but the effects on IgM were not as strong.

A preliminary analysis of IgG isotypes indicated that there was a stimulation of all isotypes of anti-TT IgG in RA and PIC-treated rats. PIC alone stimulated an increase almost exclusively of anti-tetanus IgG2b (the rat equivalent of mouse IgG2a), which is known to be stimulated by IFN-γ. RA alone and with PIC stimulated an increase in all isotypes, suggesting that RA may have stimulated the differentiation of antigen-specific cells before isotype switching, or that the specificity of PIC in inducing IgG2b was lessened in the presence of RA.

We tested the efficacy of RA and PIC treatment in a Balb/c mouse model for two reasons: first, as a means of confirming that RA and PIC are effective in a second model; second, because this model is more amenable to genetic manipulation and would therefore be of value for possible future experiments using transgenic mice. In the mouse studies we tested whether RA and/or PIC would benefit the immune system of an animal that was not made VA deficient, and therefore should not be immunocompromised. There were both similarities and differences between the results in the Lewis rat and Balb/c mouse models, as outlined in Table 4. As with the Lewis rat, RA alone elevated both the primary and secondary antibody responses of Balb/c mice during VA deficiency. However, in contrast to the Lewis rat model in which PIC only elevated the primary IgG response, in VA-deficient Balb/c mice both the primary and secondary antibody responses were enhanced. The major difference, however, was that although the combination of RA and PIC still increased antibody production, they did so in an additive, rather than synergistic, fashion (Figs. 3 and 4).

To test whether normal mice could still benefit from supplementation with RA or PIC, we included animals of adequate VA status and immunized them with TT, as for studies with VA-deficient mice. In both the primary and the secondary antibody responses RA or PIC administered alone elevated IgG levels significantly compared with untreated VA-sufficient controls ($P<0{\cdot}02$ and $P<0{\cdot}0002$ for primary and secondary antibody responses respectively; Figs. 3 and 4). When these two agents were administered together the production of anti-tetanus IgG was heightened, although we did not observe the same cooperativity as during VA deficiency.

Table 4. Comparison of the effects of retinoic acid (RA) and polyriboinosinic acid : polyribocytidylic acid (PIC) on antibody responses in rat and mouse models

Effect	Lewis rats	Balb/c mice
VA deficiency reduced antigen-specific antibody production	Yes	No
RA enhanced antigen-specific IgM and IgG levels	Yes	Yes (IgG)
PIC enhanced IgM and IgG levels	Only in the primary response	In both primary and secondary responses
RA and PIC increased IgM and IgG levels	Yes, synergistically	Yes, additively (IgG)
Total IgG increased during VA deficiency	Not significantly*	Yes
RA and PIC induced total IgG levels	Yes	Yes

Ig, immunoglobulin; VA, vitamin A.

* There was a trend in the present study to higher total IgG in VA-deficient Lewis rats; in previous studies in the same rat model this difference was statistically significant (Kinoshita *et al.* 1991).

One puzzling observation was that even second-generation VA-deficient mice were able to maintain near-normal functions with regard to antigen-specific IgG production. Mice of different genetic strains are well known to differ markedly in response to antigens, cytokines and other stimuli. Balb/c mice have been reported to have a propensity towards the T-helper 2 phenotype, associated with strong antibody production, shifting away from the T-helper 1 phenotype associated with strong cell-mediated immunity (see Mosmann & Coffman, 1989; Himmelrich *et al.* 1999). A possible explanation is that these mice were genetically poised for strong antibody production such that VA status was not a factor in their ability to respond to the immune challenge we employed. We showed previously that VA-deficient rats challenged with TT or pneumococcal polysaccharide are capable of mounting a very strong antigen-specific response, in the absence of VA or RA, if co-immunized with lipopolysaccharide (Pasatiempo *et al.* 1992; Arora & Ross, 1994). Thus, VA is not an obligatory factor for the immune response measured here, even in VA-deficient rats, but appears to be necessary in rats in the absence of other strong stimuli such as lipopolysaccharide or PIC. Moreover, a similar cooperativity was observed previously in VA-deficient rats treated with lipopolysaccharide and VA (Arora & Ross, 1994) to that in the present study following treatment with PIC and RA. Although we saw no significant effect on antigen-specific antibody production in VA-deficient mice, these mice did have low serum retinol levels, as in rats, and elevated total IgG expression, a change which was shown previously in VA deficiency (Gershwin *et al.* 1984; Kinoshita *et al.* 1991; Kinoshita & Ross, 1993).

An attractive hypothesis based on our results is that PIC elicits factors, such as type I IFN, that inhibit the activation-induced cell death of T- and B-cells, as demonstrated in cultured cells. Provision of RA may enhance the ability of antigen-activated cells to respond to IFN, for example by inducing STAT or IRF factors involved in type I and type II IFN signalling. Indeed, a significant increase in STAT-1 mRNA expression was observed in the spleen of VA-deficient rats treated with RA and PIC (DeCicco *et al.* 1999). A possible model of these interactions is shown in Fig. 5. Further studies in VA-deficient and VA-sufficient animals are planned to test and clarify this model.

In conclusion, the present study has provided evidence that RA and PIC can interact, additively or synergistically depending on the animal model, to enhance antibody production against a protein antigen. Since PIC induces the production of both type I and type II IFN, which use distinct cell-surface receptors and signalling pathways, the combination of retinoids and PIC may activate a greater response than would be elicited by a single form of IFN, by stimulating multiple pathways or factors involved in retinoid and IFN signalling. Based on the efficacy of PIC alone in several models of infection and the results reported

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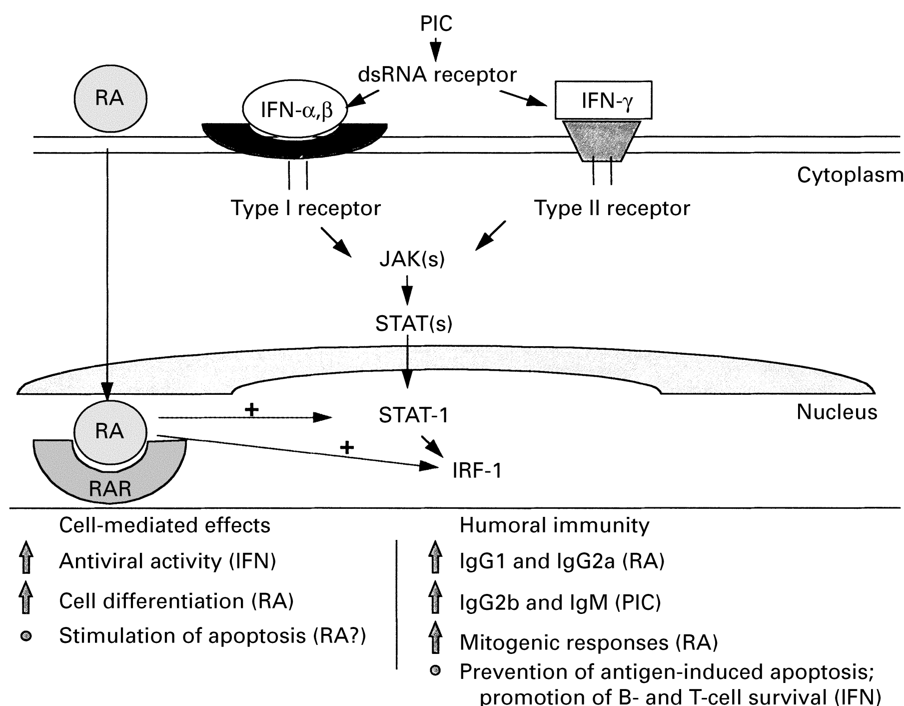

Fig. 5. Possible model for the interactions of retinoic acid (RA), interferons (IFN) and polyriboinosinic : polyribocytidylic acid (PIC) on antibody production. PIC binds to a general double-stranded (ds) RNA receptor leading to the production of IFN and other cytokines. The two major classes of IFN, type I (IFN-α and -β) and type II (IFN-γ), signal via distinct plasma membrane receptors which utilize similar but non-identical signal transduction pathways, each involving sequential recruitment and phosphorylation of Janus protein tyrosine kinases (JAK) and signal transducers and activator of transcription (STAT) family members. The phosphorylated STAT proteins migrate to the nucleus and bind as heterodimers or homodimers to the promoter regions of IFN-responsive genes. Among these IFN-responsive genes are the interferon regulatory factor family (IRF)-1,2 genes, as well as many others (not shown). These numerous anti-viral genes promote various anti-tumour activities, including enhancing apoptosis, cellular differentiation and angiogenesis. Retinoic acid (RA) exerts its regulatory actions by binding to specific nuclear receptors on target cells (RA receptors; RAR), which then associate with RA response elements to stimulate target gene expression. Some of the target genes induced by RA include STAT-1 and IRF-1, supporting the anti-viral role of RA. In addition to their effects on cell-mediated immunity, RA and PIC also affect humoral immunity. Both RA and PIC induce immunoglobulin (Ig) G subclasses (IgG1, IgG2a for RA and IgG2b for PIC), as well as IgM production. Type I IFN also affects humoral immunity, by preventing antigen-induced apoptosis and enhancing activated T-cell and B-cell survival. Since PIC induces type I IFN we speculate that it too may prolong antigen-activated cell survival, which may partially account for the enhancement in antibody production. +, positive stimulated reponse.

here, we suggest that the combination of RA and PIC should be evaluated further for its anti-infective potential.

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