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Impairment of cytokine production in mice fed a vitamin D3-deficient diet

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SUMMARY

C57Bl/6 female mice fed a Vitamin D (VIT-D)-deficient diet had serum levels of 25-hydroxyvitamin D decreasing with the time of diet exposure (3 and 8 weeks). Cytokine production (IL-6, TNF and IL-1) by peritoneal macrophages cultured *in vitro* with a standard stimulus, LPS, evaluated in the supernatants as biological activity, was significantly reduced in VIT-D-deficient animals. The defect in monokine production was partial and was evident at suboptimal LPS concentrations and incubation times. I-A antigen expression, induced in macrophages by *in vitro* exposure to IFN-γ, was not modified in VIT-D-deficient mice, but IFN-γ-inducible macrophage cytotoxicity to tumour target cells was significantly decreased in VIT-D-deficient animals. Moreover, basal and Poly I:C-induced NK activity was not modified by VIT-D deficiency. Thus, macrophage functions, such as cytokine production and tumour cytotoxicity induction, are down-modulated *in vitro* by VIT-D deprivation. To give more support to the relevance of VIT-D availability for cytokine production, TNF and IL-6 have been evaluated in the sera of control and VIT-D-deficient mice given LPS as a model stimulus. Serum peak levels of both cytokines were at least halved in VIT-D-deprived mice. Thus, VIT-D deficiency may represent a model of partial defect of monokine production.

INTRODUCTION

Vitamin D (VIT-D) plays an important role in mineral metabolism. The most active metabolite of VIT-D, 1α,25-dihydroxyvitamin D3 (1,25-OHD), has been viewed as a regulator of calcium and phosphorus metabolism at target tissues, including intestine, bone and kidney, where it acts in a manner similar to that of classical steroid hormones by binding to a specific, cytoplasmic receptor protein.[1] In addition to the classical target tissues, VIT-D-specific receptors have been discovered in nearly every vertebrate cell type,[2] including immunocompetent cells,[3] suggesting a biological role for VIT-D wider than originally believed.

It is now recognized that cells of the mononuclear phagocyte system actively interact with this vitamin. The active metabolite 1,25-OHD can induce monocytic differentiation in human[4,5] and murine leukaemic cell lines,[6] modulate differentiation, maturation and functions of authentic mononuclear phagocytes.[7-9] The active metabolite can be produced by activated macrophages that convert the relatively inactive form 25-hydroxyvitamin D3 to its most biologically active form 1,25-OHD.[10] Macrophage and polymorphonuclear leucocyte spontaneous migration and tumour cytotoxicity mediated by activated macrophages have been reported to be affected by VIT-D bioavailability.[11,12] Moreover, monocytes exposed *in vitro* to 1,25-OHD produce increased amounts of interleukin-1 (IL-1), tumour necrosis factor (TNF) and prostaglandin E_2 (PGE_2).[13-15] Thus VIT-D appears to act in immunological and inflammation processes, stimulating macrophage and neutrophil functions. Abnormal functions of phagocytes have been suggested as a possible cause of the frequent infections reported in VIT-D-deficient rachitic infants.[16]

The purpose of this study was to extend data on the interaction between VIT-D and macrophage functions, investigating the ability of macrophages from VIT-D-deprived mice to produce cytokines. IL-1, IL-6 and TNF have been chosen as products of stimulated macrophages because of the key role they play in the onset and regulation of the immune response and inflammation.[17,18] The study has been performed by inducing VIT-D-deficient state in mice, feeding them with a VIT-D-deficient diet just after weaning. Cytokine production by macrophages appeared to be sensitive to VIT-D deficiency, giving further evidence of the key role played by the vitamin in normal macrophage functions.

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MATERIALS AND METHODS

Mice

C57BL/6 female mice from Charles River, Calco, Italy were used. Weaning mice (3 weeks old) were fed a VIT-D3-deficient or control diet (Dieta Purificata DP/1017 without vitamin D3 or with 500 mg vitamin D3/kg of diet; Altromin-Rieper spa,

Vandoies, Italy) and experiments were performed 3–8 weeks later. Mice were housed five per cage in a controlled environment room with 12 hr of light per day and 60% relative humidity at 22°. Diet and municipal tap water were provided *ad libitum*. Body weight in control and VIT-D-deficient mice at 6 and 11 weeks was not different; at 6 weeks it ranged between 17·0 and 19·0 g and at 11 weeks between 21·0 and 23·0 g for both groups.

Peritoneal cells

Macrophages were obtained by lavages of the peritoneal cavity 4 days after an i.p. injection of 1·5 ml of a 3% thyoglicollate solution (Difco, Laboratories, Detroit, MI). The percentage of macrophages in these suspensions was usually 75–80%. For *in vitro* production of cytokines, macrophages were adherence-purified as described previously.[19]

Cytokine production in vitro

One-and-half million of adherence-purified peritoneal macrophages, seeded in 16-mm wells (Costar, Badhoevedorp, The Netherlands) in 1 ml of RPMI-1640 medium, were exposed to lipopolysaccharide (LPS) (0·01–1 μg/ml) (Difco) for up to 48 hr for IL-1 and IL-6 induction, and 20 hr for TNF. Incubations were performed with 1% foetal calf serum (FCS; Hyclone, Logan, UT; LPS content $< 0{\cdot}05$ ng/ml) for IL-1 and IL-6, and in the same medium without FCS for TNF induction.

Cytokine induction in vivo

Blood was obtained 0·5, 1, 2 and 4 hr after injection of LPS (2·5 μg/mouse i.p.) and IL-6 and TNF levels were evaluated in the sera by the methods described below.

IL-6

The level of IL-6 was measured on the IL-6-dependent 7TD1 cell line (Dr J. van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium), as described previously.[20] Briefly, 7TD1 were plated at 2×10^3 cells in 200 μl per well in 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark) in the presence of various dilutions of testing samples (macrophage supernatants and sera). After 3 days of incubation the growth of the cells was evaluated by the MTT method[20] and the absorbance was read at 550 nm in a spectrophotometer Titertek Multiscan MC (Flow Laboratories, Milan, Italy). The amount of IL-6 was calculated using a reference standard curve obtained in the presence of known amounts of human recombinant IL-6 (rIL-6; Genetics Institute, Cambridge, MA). One-half maximal unit of hybridoma growth factor corresponds to 20 pg/ml of rIL-6.

TNF

The level of TNF was tested on L929 cells (ATCC, Rockville, MD)[21] plated at a concentration of 1×10^4 cells per well in 96-well flat-bottomed microtitre plates (Falcon, Becton-Dickinson Labware, Oxnard, CA) and cultivated for 24 hr. Then 1 μg/ml actinomycin D (Sigma, St Louis, MO) was added 2 hr before the addition of various dilutions of testing samples (supernatants and sera). Twenty hours later plates were washed, stained with crystal violet (0·5% in methanol-H_2O, 1:4) and the absorbance was read at 550 nm in a spectrophotometer. The amount of TNF was calculated using a reference standard curve obtained in the presence of human recombinant TNF (rTNF; kind gift from W. Fiers, State University of Ghent, Ghent, Belgium). One-half maximal unit of cytotoxic factor corresponds to 20 pg of rTNF.

IL-1

The level of IL-1 was evaluated on the D.10(N4)M line (Dr L. Aarden, Central Lab. of the Blood Transfusion Service of the Netherlands Red Cross, Amsterdam, The Netherlands).[22] Briefly, supernatants were serially diluted in 96-well microplates, 1×10^4 D.10(N4)M cells were then added in RPMI-1640 medium supplemented with 3 μg/ml concanavalin A (Con A), 60 U/ml of hrIL-2 (Biogen, Geneva, Switzerland), 2-mercaptoethanol (2×10^{-5} M) and 10% FCS, and subsequentially incubated for 72 hr. Cell growth was evaluated with the MTT method as described above for the IL-6 test. Units/ml were calculated using a reference curve obtained in the presence of known amount of rIL-1β (Sclavo, Siena, Italy). One-half maximal unit of growth factor corresponds to 10 pg/ml of rIL-1β.

Macrophage-mediated cytolytic activity

The cytolytic activity of purified macrophages was evaluated after 48 hr of incubation as [^{3}H]thymidine release from prelabelled mKSA-TU5 (TU5) target cells, as described previously.[19] Macrophages were obtained from mice injected with 1 ml of phosphate-buffered saline (PBS) 24 hr before.[23] Murine recombinant interferon-gamma (IFN-γ; Ernst-Boehringer, Wien, Austria) at 3 and 15 U/ml was added at the same time as tumour target cells. At the concentrations employed, it had no activity on tumour cell viability. Results are presented as percentage of specific release of isotope.

Ia expression

The percentage of macrophages expressing Ia antigen was identified in a complement-dependent cytotoxicity assay, as previously described, using anti-I-A^b monoclonal antibodies produced by the 25.9.3S hybridoma (ATCC).[24] Briefly, macrophages (1×10^5/6·4 mm well) were exposed to 0·1 ml of hybridoma supernatant for 30 min at 4°. Subsequently the hybridoma supernatants were replaced with 0·1 ml of a 1:20 dilution of Low-Tox-M rabbit complement (Cedarlane Laboratories Ltd, Ontario, Canada) and the cultures were incubated for 45 min at 37°. Cytotoxicity was determined by trypan blue exclusion counting 100 cells/well in triplicate under a 200 × magnification.

To induce Ia antigen expression, macrophages were exposed to 100 U/ml of IFN-γ for 2 or 4 days, times when Ia expression was still increasing or had reached a maximum level, respectively.[25]

NK cell activity

Natural killer (NK) activity was evaluated as specific release of isotope from ^{51}Cr-labelled YAC-1 lymphoma cells in a 4-hr assay, as previously described,[26] using a range of attacker:target (A:T) cell ratios from 12:1 to 200:1. Poly I:C (Sigma) was dissolved in sterile pirogen-free saline and 100 μg/mouse were injected i.p. 16 hr before test.

VIT D serum levels

Circulating levels of 25-hydroxyvitamin D, the major circulating metabolite of VIT-D, were evaluated by radioimmunoassay, using a commercial kit (Nichols Institute Diagnostic, San Juan Capistrano, CA).[27,28]

Statistical analysis

Statistical significance was established by Student's *t*-test as indicated. Each type of experiment has been performed at least

Table 1. Cytokine production by peritoneal macrophages from mice fed VIT-D-deficient diet for 8 weeks

Exp. group	LPS (μg/ml)	IL-1 (U/ml)	IL-6 (U/ml)	TNF (U/ml)
Control	0·01	144±8	2460±874	ND*
w/o VIT-D	0·01	39±24†	248±112‡	ND
Control	1·0	125±66	1804±233	59±6
w/o VIT-D	1·0	121±2	387±83‡	24±3‡

$1{\cdot}5 \times 10^6$ adherence purified peritoneal macrophages were incubated with different LPS concentrations for 24 hr (IL-1 and IL-6) or 4 hr (TNF). Cytokines in supernatants were tested on D.10.(N4)M cells for IL-1, 7TD1 cells for IL-6 and Act-D-treated L929 cells for TNF.

* ND, not done; †$P<0{\cdot}05$; ‡$P<0{\cdot}01$.

three times with four to five animals per experimental group, tested individually. Results are expressed as mean±SD of values from one representative experiment.

RESULTS

VIT-D levels in the sera

To check the efficacy of the diet in inducing a state of VIT-D deficiency, sera from diet-fed and control animals were tested for VIT-D levels after 3 and 8 weeks of feeding (10 mice/group). After 3 weeks, serum levels of 25-OHD in VIT-D-deficient diet-fed mice were 10·3±2·5 ng/ml, while levels in VIT-D-supplemented mice were 34·9±3·2 ng/ml. When 25-OHD was checked in the sera of mice fed 8 weeks with the VIT-D-deficient diet, levels were 5·2±1·7 ng/ml and 43·9±2·9 ng/ml for VIT-D-deprived and control mice, respectively. Thus 3 weeks of diet can be sufficient to induce VIT-D deficiency, though levels can be further reduced after a longer exposure.

In vitro cytokine production by peritoneal macrophages

Peritoneal macrophages were taken from animals after 3 and 8 weeks of diet. Table 1 shows one typical experiment in which production of IL-1, IL-6 and TNF was evaluated after 8 weeks of VIT-D-deficient diet, under conditions in which maximal reduction of cytokine release was observed. Figures 1 and 2 summarize results from the whole series of experiments, in which cytokine production was evaluated after 3 and 8 weeks of diet, with different LPS concentrations and incubation times. After 3 weeks of VIT-D-deficient diet there were no alterations in the ability of macrophages to produce biologically active IL-1 in supernatants after stimulation with either LPS concentration (0·01–1 μg/ml) (Fig. 1a). Mean values for VIT-D-deprived mice at 24 and 48 hr of incubation with 0·01 μg/ml were lower than controls, but variability was always high and the differences were never significant. When feeding time was increased to 8 weeks, macrophages from VIT-D-deficient mice incubated with 0·01 μg/ml LPS showed a decreased production of IL-1 after 24 hr but when incubation was prolonged for 48 hr, levels similar to controls were obtained for VIT-D-deficient mice (Fig. 1a). With the highest LPS concentration (1 μg/ml), macrophages from mice fed the VIT-D-deficient-diet were able to produce levels of IL-1 similar to controls animals at 24 and 48 hr, showing that the ability of macrophages from VIT-D-deficient mice to produce IL-1 was only partially affected.

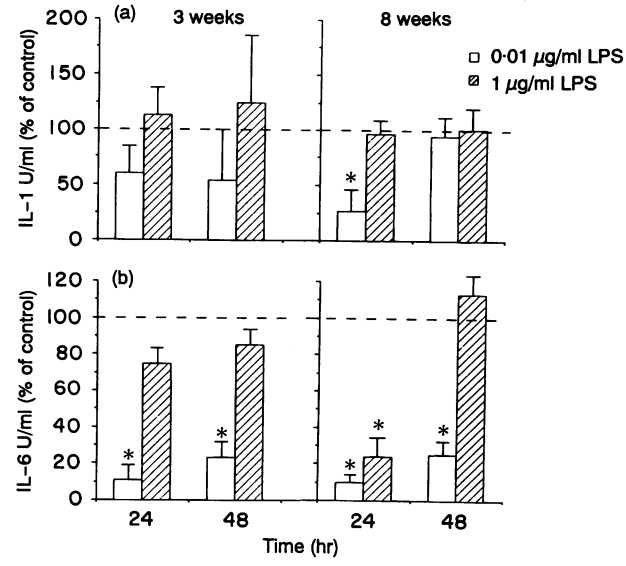

Figure 1. IL-1 (a) and IL-6 (b) production by peritoneal macrophages from mice fed control or VIT-D-deficient diet for different times. $1{\cdot}5 \times 10^6$ adherence purified macrophages were cultured for times reported (24 and 48 hr) in 1 ml of RPMI-1640 medium + 1% FCS and 0·01 or 1 μg/ml of LPS. IL-1 and IL-6 in supernatants were tested on D.10.(N4)M and 7TD1 cell lines, respectively. Values for control mice at each time point are reported here as 100% (dotted line). *$P<0{\cdot}01$.

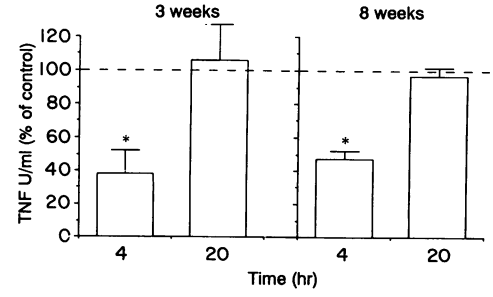

Figure 2. TNF production by peritoneal macrophages from mice fed control or a VIT-D-deficient diet for different times. Macrophages were cultured from 4 to 20 hr in RPMI-1640 medium in the presence of 1 μg/ml LPS. TNF in supernatants was tested on the Act-D-treated L929 cells. TNF values for control mice at each time-points are reported here as 100% (dotted line). *$P<0{\cdot}01$.

The production of IL-6, a macrophage product which shares many properties with IL-1 except inflammatory activities, was investigated next. Figure 1b shows that after 3 weeks IL-6 production was significantly inhibited at 24 and 48 hr, when the LPS concentration was 0·01 μg/ml; levels of IL-6 were highly reduced, being only 11% and 23% of controls at 24 and 48 hr, respectively. When LPS concentration was 1 μg/ml, a slight reduction in IL-6 levels (15–25%) was consistently observed, but was never statistically significant. After 8 weeks of diet, IL-6 production was reduced as at 3 weeks with the lower concentra-

Table 2. Effect of IFN-γ on Ia antigen expression by macrophages from mice fed VIT-D-deficient diet for 8 weeks

Exp. group	IFN-γ (U/ml)	% I-A$^+$ macrophages 0*	2	4
Control	—	30·6+3·2	12·0+1·5	7·0+1·0
w/o VIT-D	—	32·7+4·0	8·0+2·9	6·0+2·5
Control	100	ND†	74·5+4·6	82·0+2·0
w/o VIT-D	100	ND	72·3+6·9	81·0+2·6

1×10^5 macrophages were exposed to anti-I-A^b monoclonal antibodies and complement just after collection (Day 0) or after 2 and 4 days of *in vitro* culture with or without IFN-γ. Cytotoxicity was determined by trypan blue exclusion.

* Day of culture.

† ND, not done.

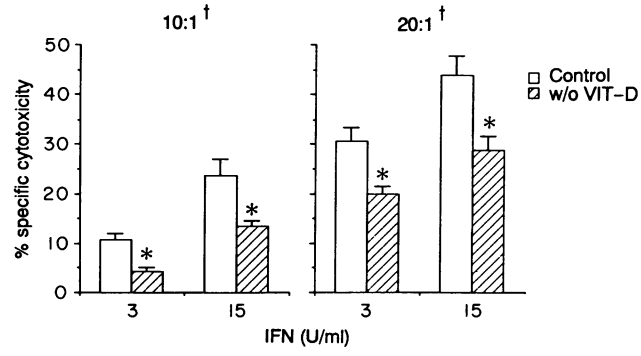

Figure 3. Induction of macrophage-mediated cytotoxicity against tumour target cells by IFN-γ. Adherence-purified peritoneal macrophages from mice fed 8-week control or a VIT-D-deficient diet were incubated with TU5 prelabelled tumour target cells for 48 hr in the presence or absence of IFN-γ at concentrations reported. Macrophages from both experimental groups not exposed to IFN-γ had no detectable cytotoxic activity. † Attacker:target (A:T) cells ratios of 10:1 and 20:1 have been employed. *$P < 0{\cdot}01$.

tion of LPS (Fig. 1b and Table 1). Moreover, significant inhibition was observed after exposure of macrophages to 1 μg/ml LPS for 24 but not 48 hr. Thus, IL-6 production seems to be more susceptible to VIT-D deficiency than IL-1, since it was already significantly reduced after 3 weeks and inhibition could be further increased after 8 weeks, as indicated by the data obtained with 1 μg/ml LPS.

To further characterize the effects of VIT-D deficiency on macrophage ability to produce soluble factors, TNF production was followed after incubation of macrophages for 4–20 hr with 1 μg/ml LPS. Results are reported in Fig. 2 and Table 1. TNF production was significantly reduced at 4 hr, but reached the levels of controls at 20 hr. The degree of inhibition of TNF production was essentially the same after 3 or 8 weeks of VIT-D deprivation. The ability of macrophages to produce cytokines, especially TNF and IL-6, was clearly impaired when animals were deprived of VIT-D, extending available data on the impairment of macrophage functions by VIT-D deficiency.[11,12]

In vitro activation of macrophages by IFN-γ

Having established that macrophages from VIT-D-deficient mice have defective responsiveness, in terms of monokine production, to a bacterial product, it was important to investigate their ability to respond to IFN-γ, a prototypic lymphokine active on mononuclear phagocytes. We studied tumour cytotoxicity and class II MHC antigen expression after *in vitro* exposure of macrophages to IFN-γ. Results in Table 2 show that macrophages from mice after 8 weeks of VIT-D-deficient diet expressed Ia-antigen basal levels similar to controls. After exposure to IFN-γ (100 U/ml) for 2 or 4 days, Ia expression increased to the same levels in macrophages from control and VIT-D-deficient mice. There were also no differences in Ia expression in macrophages not exposed to IFN-γ, where the percentage of Ia-positive macrophages decreased in culture at the same level in control and VIT-D-deficient mice.

Results on induction of cytotoxicity are reported in Fig. 3. Macrophages from mice fed a VIT-D-deficient diet for 8 weeks were defective in their ability to respond to IFN-γ; in fact the level of induced cytotoxicity was about half that reached by control macrophages. Cytotoxicity of macrophages not exposed to IFN-γ was negligible in both groups of animals.

Thus, macrophages from VIT-D-deficient animals were able to express normal level of Ia antigens after *in vitro* incubation with IFN-γ, but displayed a reduced ability to respond to the same stimulus as regards activation for tumour cytotoxicity.

NK activity

Since NK cells have been proposed to be related to the monocyte–macrophage lineage and VIT-D deficiency was able to modify macrophage functions, it was of interest to investigate the effect of VIT-D deprivation on the activity of NK cells. As shown in Fig. 4, VIT-D deprivation did not affect NK activity and its stimulation by Poly I:C.

Cytokine production *in vivo*

From the results obtained, it was clear that macrophage functions, at least when evaluated *in vitro*, were substantially modified by VIT-D deprivation. Because of the key role played by cytokines such as IL-6 and TNF in different immunological and inflammatory situations, it was important to evaluate the *in vivo* relevance of these *in vitro* observations. Mice were challenged with LPS as stimulus for cytokine production, and circulating levels of IL-6 and TNF evaluated at different times. Results obtained in mice after 3 or 8 weeks of diet are reported in Fig. 5.

IL-6 serum levels in mice after 3 or 8 weeks of diet were significantly decreased (50%) at 2 hr after LPS injection, that is at the peak time for this cytokine, without consistent difference at any other time investigated (Fig. 5a). TNF serum levels in VIT-D-deprived mice were significantly lower than controls 0·5 and 1 hr (peak time) after LPS injection (Fig. 5b). Similar effects have been obtained at 3 and 8 weeks.

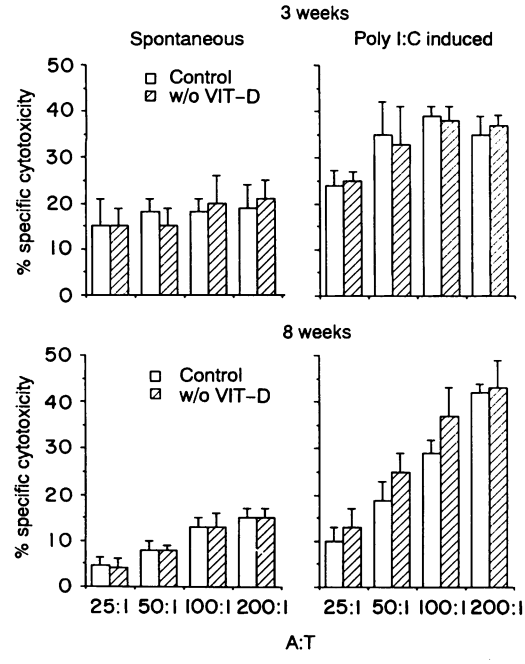

Figure 4. NK activity of splenocytes from mice fed control or VIT-D-deficient diet for different times. Mice were injected i.p. with sterile pyrogen-free saline or Poly I:C (100 μg/mouse) 16 hr before test. A:T = attacker:target cells ratio.

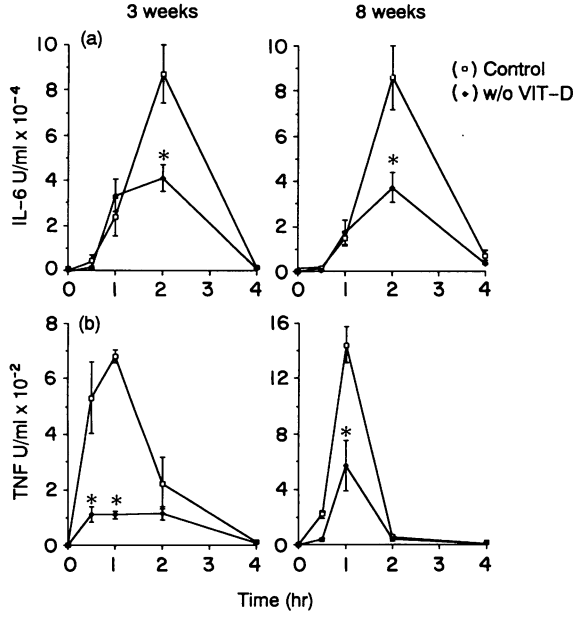

Figure 5. IL-6 (a) and TNF (b) levels in sera of LPS injected mice. Sera were collected at times reported (hr) after an i.p. injection of 2·5 μg LPS/mouse. $*P < 0{\cdot}01$.

Inhibition of cytokine production in VIT-D-deprived mice was thus confirmed to be a consistent phenomenon that also occurred *in vivo*.

DISCUSSION

The results presented here demonstrate that mice fed a VIT-D-deficient diet had macrophages with a defective capacity to release the cytokines IL-6, TNF and IL-1. By and large, the defect in monokine production was not absolute and was evident at suboptimal LPS concentrations and incubation times. Also, defective monokine production was better evident for IL-6 and TNF than for IL-1. The *in vivo* relevance of these *in vitro* observations was examined by injecting mice with LPS: VIT-D-defective mice showed significantly lower levels of IL-6 and TNF upon challenge with this model stimulus. These observations extend previous studies on the importance of VIT-D in the regulation of mononuclear phagocyte functions. Phagocytes from mice fed a VIT-D-deficient diet have been shown to have defective migratory capacity *in vitro* and *in vivo*[11,12] and *in vitro* exposure to 1,25-OHD augmented the chemotactic responsiveness of monocytes.[9] Moreover monocytes exposed to VIT-D3 *in vitro* produced increased amounts of IL-1 and TNF.[13,14]

It has been reported recently that macrophages from VIT-D-deficient mice have defective cytotoxicity on a TNF-sensitive (L929) cell line.[12] The defective production of TNF demonstrated here probably underlies this observation. In the present study, macrophage cytotoxicity was studied using a TNF-resistant cell line[29] and was found to be defective in VIT-D-deficient animals. Thus, defective effector function is not confined to the TNF-mediated cytotoxicity pathway of macrophages.

The functional alterations of mononuclear phagocytes from VIT-D-deficient mice were selective and did not imply a generalized deficiency of this cell population. In fact, macrophages from VIT-D-deficient mice, upon exposure to IFN-γ, expressed normal levels of class II MHC antigens. Interestingly, the same stimulus, IFN-γ, that efficiently induced Ia, elicited defective levels of tumour cytotoxicity in VIT-D-deficient macrophages, an observation which further emphasizes the selectivity of the impact of VIT-D deficiency on macrophage function.

The controversial suggestion that NK cells can be immature elements in the myelomonocytic differentiation pathway[30,31] and the fact that VIT-D modulates mononuclear phagocyte differentiation, prompted us to investigate the effects of VIT-D deprivation on NK cell functions. No differences in the basal and Poly I:C-induced NK activity have been found after 3 and 8 weeks of diet, weakening the hypothesis of NK cells as monocyte precursors. It cannot be excluded, however, that VIT-D deficiency does not affect the cytotoxic ability of cells that are still immature elements, and that the defect can become evident when functions of fully differentiated cells are investigated.

VIT-D-deficient rickets has long been known to be associated with augmented susceptibility to infection. Monokines play a central role in inflammation and immunity and exogenously administered IL-1 and TNF have been demonstrated to enhance resistance to bacterial infections.[32,33] It is therefore likely that diminished production of IL-1, IL-6 and TNF

contributes to the immunodeficiency caused by VIT-D deprivation.

VIT-D deficiency may represent a novel experimental model of defective monokine production, useful to investigate the role played by these mediators in physiological and pathological processes.

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