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The importance of interferon-γ in an early infection of *Chlamydia psittaci* in mice

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SUMMARY

Athymic mice (nu/nu) and their hairy littermates (nu/+) were infected experimentally with *Chlamydia psittaci* and the role of endogenous interferon-γ (IFN-γ) on the resolution of the infection was studied. The pathological changes produced in the spleen, liver and lung were exacerbated by administration of a monoclonal antibody (mAb) to IFN-γ and an increased number of viable chlamydiae were recovered from the tissues of both nu/+ and nu/nu mice treated in this way.

INTRODUCTION

Chlamydia psittaci is the causative agent of enzootic abortion of ewes as well as of respiratory diseases in birds and man. Studies of chlamydial immunity have shown that lymphokines can restrict the growth of *C. psittaci* in murine macrophages *in vitro*[1] and that the inhibition of growth can be reversed by adding anti-interferon-γ (IFN-γ) antibody to the culture.[2,3] In addition, the ability of IFN-γ to activate the microbicidal activity of host macrophages,[4] as well as cause suicide-like cell destruction of bacteria-infected cells when triggered by bacterial lipopolysaccharide (LPS),[5–7] may be important in host resistance to *C. psittaci*. Recent *in vivo* studies have also demonstrated a protective role for IFN-γ in *C. trachomatis* infections.[8–10] The following study was designed to investigate the *in vivo* effects of endogenous IFN-γ on the resolution of a *C. psittaci* infection in mice.

MATERIALS AND METHODS

Chlamydia psittaci

The S26/3 ovine abortion strain of *C. psittaci* was grown in the yolk sac of fertilized hens' eggs. Titration of inoculum was carried out by adding serial dilutions of *C. psittaci* to baby hamster kidney cell monolayers[11] and counting the number of inclusions formed.[12] Results are expressed as inclusion-forming units (IFU).

Animals

Athymic nude mice (nu/nu) on a MF1 background and their hairy littermates (nu/+) (Harlan Olac Ltd, Oxford, U.K.) were divided into eight groups containing between four and 12 mice (Table 1) and maintained in positive pressure isolators for the duration of the experiment. Group A consisted of nu/+ mice infected intraperitoneally (i.p.) with 10^6 IFU of *C. psittaci*. Group B consisted of nu/+ mice infected with the same dose of *C. psittaci* and then injected i.p. with monoclonal antibody (mAb) to IFN-γ on days −1, 0, 1 and 3. On each occasion each mouse received 200 μl of mAb which neutralized 10^5 units of murine IFN-γ. Groups C and D comprised nu/nu mice and were treated in the same way as groups A and B respectively.

Control groups E and F consisted of uninfected nu/nu and nu/+ mice respectively and were treated with the mAb in the same manner as groups B and D. Groups G and H consisted of nu/nu and nu/+ mice respectively and were given a control inoculum of 450 μl of uninfected egg yolk sac on day 0.

Monoclonal antibody

The rat anti-mouse IFN-γ mAb designated R46A2[13] was produced by growing the rat/mouse hybridoma cells in the peritoneal cavity of athymic mice. The ascitic fluid neutralized murine IFN-γ and the titre was determined by bioassay.

Procedure

In each group, half of the mice were killed with CO_2 on day 3 and the other half on day 5. Animals were weighed and blood was collected so that sera could be analysed for IFN-γ activity. Macroscopically lesions were recorded and using aseptic precautions, the spleen from each animal was removed and weighed before it and samples of liver and lung were taken for attempted isolation of *C. psittaci*[12] and for histopathological studies. The latter samples were fixed immediately in 10% formal saline while samples for isolation were frozen at −70° in chlamydial transport medium.

Histopathology

Tissues were fixed for 2–4 hr and selected blocks processed to paraffin wax. Sections, 5 μm thick, were cut and stained with haematoxylin and eosin (H&E) while adjacent serial sections were stained with an immunoperoxidase method to demonstrate chlamydial antigen.[14]

Liver histopathology

The pathological changes in liver sections stained by H&E were quantified.

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Table 1. The group sizes and treatments used to evaluate the role of endogeneous IFN-γ in the resolution of a live chlamydial infection in mice

Group	*n*	Type	Treatment	Day killed
A	5	Nu/+*	*C. psittaci* only‡	3
	5			5
B	5	Nu/+*	*C. psittaci*+antibody‡§	3
	5			5
C	6	Nu/Nu†	*C. psittaci* only‡	3
	6			5
D	6	Nu/Nu†	*C. psittaci*+antibody‡§	3
	6			5
E	3	Nu/Nu†	Antibody only§	3
	3			5
F	3	Nu/+*	Antibody only§	3
	3			5
G	3	Nu/Nu†	Yolk sac¶	3
	3			5
H	2	Nu/+*	Yolk sac¶	3
	2			5

* Thymic mice on a MF1 background.
† Athymic MF1 littermates.
‡ Mice given 10^6 IFU of *C. psittaci* i.p.
§ Mice given murine IFN-γ neutralizing mAb.
¶ Mice given uninfected egg yolk sac as a control inoculum.

Frequency of inflammatory foci. The inflammatory foci present in five randomly selected fields (magnification × 40) of a liver section from each mouse were counted and the mean number of inflammatory foci/field ± SE for each infected group on each day calculated.

Size of inflammatory foci. The mean number of cells in five randomly selected inflammatory foci (magnification × 160) in each mouse was counted and expressed as the mean number of cells/focus ± SE for each infected group on each day.

Percentage of cells containing antigen within an inflammatory focus. The percentage ± SE of cells containing antigen for each group on days 3 and 5 was obtained by counting 100 inflammatory cells from each of five randomly selected foci from a liver section from each mouse (magnification × 160), stained by immunoperoxidase.

Interferon assays

The IFN assay used was an adaptation of that described for ovine IFN,[15] with the exception that the target cells were murine L929 cells. Recombinant murine IFN-γ, expressed in Chinese hamster ovary cells (kindly supplied by Dr A. G. Morris, University of Warwick, Warwick, U.K.), served as a positive control. Specificity was established using the rat IgG mAb R46A2 which neutralizes murine IFN-γ.[13]

Statistical analysis

Statistical analysis on all data was carried out using Student's *t*-test.

Table 2. Results of the examination of the spleens of the infected groups A to E

Group	Treatment†	Day	% weight of spleen	Infectivity (IFU × 10^5) mean ± SE
A	Nu+/Cp	3	0·41 ± 0·04	5·6 ± 0·8
		5	0·85 ± 0·09[ac]	0·1 ± 0·1[a]
B	Nu+/Cp/Ab	3	0·62 ± 0·03[b]	9·0 ± 0·6
		5	1·01 ± 0·01[ac]	13·0 ± 4·0[a]
C	NuNu/Cp	3	0·46 ± 0·02	8·3 ± 1·1
		5	0·68 ± 0·06[ac]	13·0 ± 1·1[a]
D	NuNu/Cp/Ab	3	0·71 ± 0·03[b]	19·0 ± 1·6
		5	0·88 ± 0·06[ac]	46·0 ± 2·5[a]
E	NuNu/Cp	3	0·40 ± 0·03	0
		5	0·43 ± 0·06	0

† Nu+, thymic mice; NuNu, athymic mice; Cp, infected with *C. psittaci*; Ab, treated with anti-IFN-γ antibody.
[a] $P < 0·05$ when compared with day 3 results of same group; [b] $P < 0·05$ when compared with control day 3 results; [c] $P < 0·05$ when compared with control day 5 results.

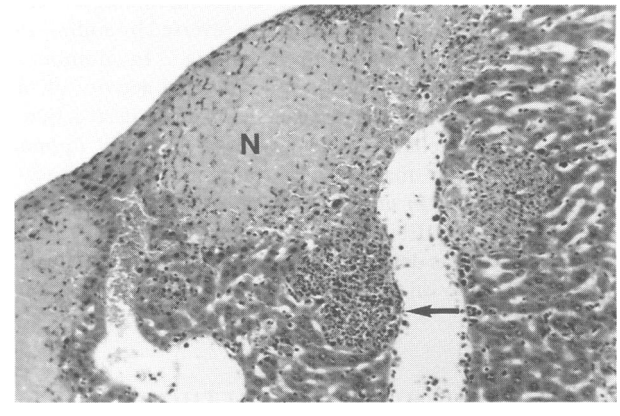

Figure 1. A section of liver tissue from a thymic mouse in group B sampled on day 5. Note the large areas of necrosis (N) and the smaller area of focal inflammation (arrow). Magnification × 38·5.

RESULTS

The control groups (E–H) showed no abnormalities. No chlamydial antigen was detected by immunocytochemistry and no chlamydiae were isolated from any tissue. Spleen size also remained constant. Therefore, data on the control groups have been omitted from all tables with the exception of group E which are included in Table 2 as a comparison.

Spleen

Weights

All mice used in the experiment were weighed on the day of kill. There was no significant weight fluctuation during the experiments when infected animals were compared with control groups, therefore spleen weights are given as per cent body weight (Table 2). On day 3 those of mice in groups B and D were

Table 3. Results of the examination of the livers of the infected groups A to D

Group	Treatment*	Day	Focal necrosis	Inflammation		% cells[a] containing Ag	Infectivity (IFU × 10^3) mean ± SE
				Foci/field	Cells/focus		
A	Nu+/Cp	3	2/5	0·64 ± 0·16	40·2 ± 5·8	3·6 ± 1·0	3·3 ± 0·2
		5	0/5	1·84 ± 0·20[b]	112·2 ± 19·0[b]	10·8 ± 2·9[b]	0·2 ± 0·3[b]
B	Nu+/Cp/Ab	3	2/5	1·0 ± 0·17	57·8 ± 6·0	7·4 ± 2·1	7·4 ± 0·2
		5	5/5	4·24 ± 0·35[bc]	129·6 ± 17·0[b]	23·6 ± 2·9[b]	32·0 ± 6·0[b]
C	NuNu/Cp	3	6/6	1·0 ± 0·16	41·6 ± 4·4	4·0 ± 1·3	7·3 ± 0·5
		5	2/6	1·43 ± 0·14[b]	88·2 ± 11·8[b]	15·0 ± 4·3[b]	3·7 ± 0·3[b]
D	NuNu/Cp/Ab	3	2/6	1·23 ± 0·19	55·8 ± 5·8	8·0 ± 1·6	16·0 ± 2·0
		5	6/6	2·70 ± 0·30[b]	206·0 ± 28·0[bc]	32·6 ± 6·1[bc]	51·0 ± 8·5[b]

* Nu+, thymic mice; NuNu, athymic mice; Cp, infected with *C. psittaci*; Ab, treated with anti-IFN-γ antibody.
[a] Percentage of inflammatory cells containing antigen; [b] $P < 0·05$ when compared with day 3 results in same group; [c] $P < 0·05$ when compared with day 5 results of all groups.

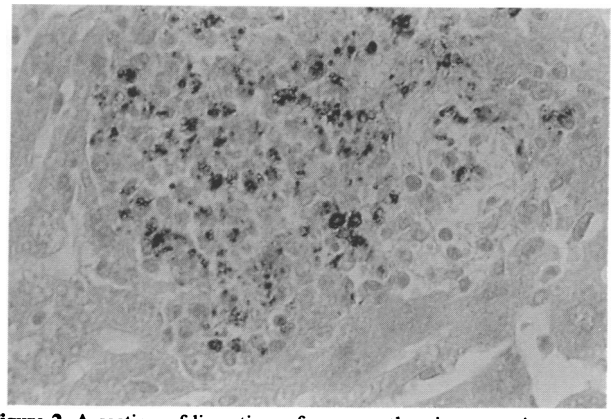

Figure 2. A section of liver tissue from an athymic mouse in group C killed on day 5. The photograph shows the detection of chlamydial antigen within a focus of inflammation by an immunoperoxidase method (IP). Magnification × 385.

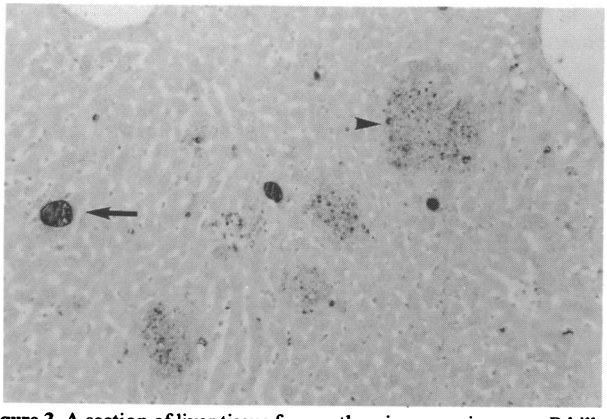

Figure 3. A section of liver tissue from a thymic mouse in group B killed on day 5. Note that as well as the detection of antigen within the foci of inflammation (arrowhead) there are also chlamydial inclusions present within the section (arrow). Magnification × 98.

significantly heavier than those in groups A and C and the control groups ($P < 0·01$). On day 5, groups A to D weights were all significantly greater ($P < 0·02$) than on day 3 and were significantly greater than those of the control groups ($P < 0·008$). Spleens in group B were significantly heavier than those in infected groups ($P < 0·003$).

Histopathology

In groups A to D periarteriolar lymphoid sheaths were more prominent at days 3 and 5 than in control groups. There was no discernible difference within any group between days 3 and 5. Vacuoles, present in the red and white pulp, contained pyknotic cells and cytolytic debris, and were more frequent in groups A and B than in groups C and D. Polymorphonuclear cells (PMN) were present in groups A to D, but more so in groups C and D.

Antigen detection. Antigen was detected in groups A to D on both days. It was mainly associated with the vacuoles described above, although a lesser amount of antigen was detected in the red pulp in the form of elementary body (EB) inclusions. The amount detected in groups A and B was similar on days 3 and 5, but less than in groups C and D where it was greater on day 5 than day 3. More inclusions were present in groups C and D than in A and B.

Isolation

Chlamydia psittaci was isolated from all spleens in groups A to D (Table 2) but the number recovered was least in group A at day 3 (lower than groups B and D; $P \leqslant 0·01$). On day 5 fewer chlamydiae were isolated from group A than on day 3 ($P \leqslant 0·002$) and fewer than from any other group at day 5 ($P \leqslant 0·03$). In group C there was little difference in the numbers isolated on days 3 and 5. More chlamydiae were recovered from group D than from any other group at day 3 ($P \leqslant 0·001$) and day 5 ($P \leqslant 0·0005$) and, within group D, more were isolated on day 5 than on day 3 ($P \leqslant 0·002$).

Liver

Macroscopic findings

White foci or streaks, usually 1–2 mm across and often bordered by a thin haemorrhagic zone, were observed in several mice in groups A to D.

Table 4. Results of the examination of the lung tissue of the infected groups A to D

Group	Treatment*	Day	Alveolar hypercellular	Hypercellular cell type mono/PMN†	Antigen	Infectivity (IFU $\times 10^3$) mean $\pm$ SE
A	Nu+/Cp	3	+	+/−	−	1·1 ± 0·2
		5	+	+/+	−	0·3 ± 0·1[b]
B	Nu+/Cp/Ab	3	+	+/−	−	4·2 ± 0·3
		5	+	+/−	+	14·0 ± 2·4[b]
C	NuNu/Cp	3	+	+/−	−	1·6 ± 0·3
		5	+	+/+	−	3·7 ± 0·5[b]
D	NuNu/Cp/Ab	3	+	+/+	−	7·8 ± 0·3
		5	+	+/+	+	36·0 ± 5·1[b]

* Nu+, thymic mice; NuNu, athymic mice; Cp, infected with *C. psittaci*; Ab, treated with anti-IFN-γ antibody.

† Monocytes/polymorphonuclear cells.

[b] $P < 0{\cdot}05$ when compared with the day 3 results of the same group.

In group A, these lesions were present in two of five mice on day 3, but none at day 5. In group B, on day 3, two of five mice were affected and on day 5 all five mice were affected. In group C, all six mice on day 3 and two of six on day 5 had lesions while in group D two on day 3 and all mice on day 5 were affected.

Histopathology

Focal necrosis. Microscopic examination of the foci showed that within them the hepatocytes and the sinusoidal lining cells had undergone coagulative necrosis and at the interface with adjacent normal tissue there was often a zone of haemorrhage (Fig. 1). Within the foci there were no inflammatory cells or significant demonstrable antigen.

Focal inflammation. A consistent finding was multiple small foci of inflammation (Fig. 1), but with little damage to the surrounding hepatocytes. Distinct from the foci of necrosis chlamydial antigen, both extracellularly and within inflammatory cells, was readily demonstrated in many of them.

The frequency of inflammatory foci in groups A to D was similar on day 3 (Table 3), but higher on day 5 ($P \leqslant 0{\cdot}001$). On day 5, there were more foci of inflammation in groups B and D than in groups A and C ($P \leqslant 0{\cdot}03$). On day 3, groups A and C had fewer inflammatory cells in each focus than groups B and D ($P \leqslant 0{\cdot}05$). In all groups the number of cells in each focus was higher on day 5 ($P \leqslant 0{\cdot}001$). At this time groups B and D had more cells in each focus than any other group ($P \leqslant 0{\cdot}04$).

Antigen detection. Antigen was observed in the livers of mice in groups A to D sampled at day 3. On day 5, more antigen was detected in all groups ($P \leqslant 0{\cdot}04$), with the largest difference in chlamydiae detected being found in groups B and D (Table 3). The antigen was generally intracytoplasmic and within the foci of inflammation (Figs 2 and 3), particularly those with large infiltrates of PMN. Mice given mAb, groups B and D, had more antigen detected on day 5 compared with groups A and C ($P \leqslant 0{\cdot}04$) and more chlamydial inclusions (Fig. 3).

Isolation

At day 3, mice in group D contained more organisms ($P \leqslant 0{\cdot}009$) than mice in group B (Table 3), as did group C when compared with group A ($P \leqslant 0{\cdot}005$). Groups B and D both showed an increase between days 3 and 5 ($P \leqslant 0{\cdot}02$) while in both groups A and C a decrease was detected in the number of organisms isolated ($P \leqslant 0{\cdot}0007$).

Lung

Histopathology

Inflammation. Groups A to D showed an increased cellularity of the alveolar septa when compared with control groups (Table 4). This was in part due to the presence of PMN, particularly in groups C and D. PMN were detected in group B on both days and on day 5 in group A. Alveolar macrophages were more frequent in groups A to D on both days when compared with controls.

Antigen detection. Antigen was only detected on day 5 in groups B and D.

Isolation

Chlamydiae were isolated from groups A to D on both days 3 and 5. In group A the number was less on day 5 than on day 3 ($P \leqslant 0{\cdot}007$), but in all other groups the numbers were greater on day 5 ($P \leqslant 0{\cdot}02$). More were isolated from groups B and D than from groups A and C (day 3 $P \leqslant 0{\cdot}0006$; day 5 $P \leqslant 0{\cdot}007$) and more were isolated from groups C and D than from A and B on both days ($P \leqslant 0{\cdot}005$; $P \leqslant 0{\cdot}006$).

Interferons

Anti-viral activity was detected only in the sera of mice from groups B and D. In group B, IFN was detected in the sera of all five mice on day 3 (titre of 120 ± 25 U/ml; mean ± SE), but the serum of only one mouse was positive on day 5 (80 U/ml). In group D sera from all six mice on day 3 were positive for IFN activity (140 ± 16) and five of six on day 5 were positive (60 ± 10).

All positive sera were then tested for the presence of IFN-γ. All sera proved to be negative for IFN-γ with the exception of one mouse from group D, which had a titre of 160 U/ml on day 5.

DISCUSSION

In all tissues examined from groups A to D, although similar histological changes were seen, treatment with the mAb against IFN-γ altered both the severity and the rate of development of these lesions. In both groups B and D, mAb treatment resulted in the earlier appearance of and the development of greater numbers of inflammatory foci in the liver. The numbers of cells in each focus were also greater. In addition, the numbers of vacuoles in the spleen decreased with time in untreated mice but not in mAb-treated groups. It is likely that these vacuoles represent sites of phagocytosis of infected macrophages, as they contained both cell debris and chlamydial antigen. It would appear therefore that in the livers and the spleens of both thymic and athymic mice not given antibody, the infection was being resolved, while in mAb-treated mice the infection was not being so readily controlled. This resulted in further tissue damage, suggesting that IFN-γ has an role to play in the control of chlamydial infection.

Infection caused a significant rise in spleen weights compared with the uninfected controls. In addition, mAb-treated mice showed the greatest increase, suggesting that the treatment resulted in an increase in blastogenesis. This effect could be due to the removal of suppressive effects that IFN-γ might exert on proliferation, as it has been identified as a potent suppressor of proliferation *in vitro*.[16] *In vitro* studies have also shown that activated splenic macrophages can suppress the proliferative response of lymphocytes to mitogens.[17] The results presented above may be an *in vivo* demonstration of these experimental phenomena.

Necrosis, visible to the naked eye, was present in the livers of infected animals and IFN-γ appeared to be associated with its onset and severity, as depletion of IFN-γ delayed the appearance of necrotic tissue until day 5 in groups B and D. The coagulative necrosis had the appearance of being the result of infarction. It is possible therefore, that chlamydial infection and its resolution caused the release of cell debris which in turn blocked hepatic blood vessels and led to necrosis.

Cell death in mouse and rat fibroblasts may be directly caused by IFN-γ[6] and enhanced by bacterial LPS.[7] This cytotoxic reaction may be protective, as infected cells would be destroyed, releasing non-infectious chlamydial reticulate bodies. Cell debris from damaged tissue may also attract phagocytic cells and aid the clearance of the organisms. The lack of antigen within the macroscopically visible foci of necrosis, however, suggests that a direct effect on the liver, as just described, is unlikely.

Another feature of the pathology of infection was the presence of foci of inflammation within the liver. These foci increased both in number and in size, on day 5 when compared with day 3. Monoclonal antibody-treated groups B and D had more and larger foci than groups A and C and larger numbers of chlamydiae were recovered from the tissue. Tissue damage and cell debris caused by the heightened infection would in turn attract more phagocytic cells and create large foci of inflammation. This hypothesis is strengthened by the finding that most of the detectable antigen was associated with these foci. Thus it seems likely that infection of Kupffer cells within the hepatic sinusoids was the first step in the formation of these foci.

Antigen detection varied between mice which had been given mAb and those which had not. In the spleen, antigen was present in cytolytic vacuoles in all groups, but in mAb-treated groups, inclusions were also present, indicating that chlamydial replication was taking place at these sites. A similar picture was seen in the liver where inclusions were seen in hepatocytes of mAb-treated mice. These inclusions were not associated with inflammatory cell infiltration, suggesting therefore that IFN-γ has a role to play in inhibiting the growth of *C. psittaci* in tissues.

The sera from groups A to D were tested for the presence of IFN, but as in previous reports IFN was not detected in the sera of infected mice not treated with mAb.[8,9] Sera from mAb-treated mice did contain IFN of the αβ type. It is not clear why IFN-αβ was produced, but it is known that many intracellular bacteria such as *Listeria monocytogenes* and *Brucella abortus* can induce IFN-αβ after systemic infection.[18,19] Therefore its production may be an indication of the extent of exacerbation of the pathology caused by the mAb. LPS is known to induce IFN-αβ in murine macrophages[20] and it is also possible that the LPS from the increased multiplication of *C. psittaci* may have stimulated this process. One mouse, treated with mAb, had IFN-γ in its serum, but the reason for this is unclear. This mouse also had less detectable antigen and harboured fewer chlamydiae than other mice in its group.

Infected mice, treated with mAb, consistently developed more severe lesions and harboured a greater number of chlamydiae in the spleen, liver and lung, compared with infected control mice. At present however, the role of T cells is uncertain. It has been shown that protection against *C. psittaci* in mice can be conferred by Lyt-2$^+$ T cells.[21] While in this study there was often a slight exacerbation of infection in athymic mice, the differences were not always significant, an exception being the greater numbers of chlamydiae isolated from the tissues of the athymic mice. Therefore it would appear that T cells also have a part to play in the clearance of a chlamydial infection. While this function has yet to be elucidated, it is clear that IFN-γ plays a major role in the early immune response to *C. psittaci*.

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