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In vivo and in vitro effect of cyclophosphamide on *Borrelia duttoni*

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In memoriam Oscar Felsenfeld

Summary

Sera taken from mice given cyclophosphamide 75 min previously fails to substantially inhibit borrelial multiplication in vitro. Mice given cyclophosphamide were infected with *Borrelia duttoni*. Inability to demonstrate specific antibody in these mice did not prevent recovery from infection, resolution of the bacteraemia or appearance of an antigenic variant. Cyclophosphamide may delete suppressor lymphocytes allowing enhanced production of antibodies which may not only protect, but induce antigenic variation. Alternatively, recovery from infection may be unrelated to the immune response.

Key words: Borrelia duttoni; antigenic variation; antibody; cyclophosphamide.

Introduction

Specific antibody appears in relapsing fever when the bacteraemia is greatest and may be responsible for terminating the infection (Wright and Ginger, 1973). Antibody may prevent the borreliosis developing (Calabi, 1959) or abort an attack if passively transfused to a patient with the active disease (Balteanu et al., 1948). Alternatively as in trypanosomiasis (Vickerman, 1974), antibody might induce antigenic variation thereby perpetuating the infection. To determine which was the dominant role of antibody, experimental infections were studied in mice whose antibody production had been suppressed by cyclophosphamide.

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Materials and methods

Organisms. The isolates of *B. duttoni* used were the Wellcome mouse adapted strain, originally obtained from Dr. J. D. Fulton at the National Institute for Medical Research, London, and a human pathogenic strain from Professor R. Geigy at the Swiss Tropical Institute, Basle (their reference number STIB 503). This strain had been repeatedly passaged in mice and ticks. Isolates were prepared from this strain either after one mouse passage (type 1) or after passaging through the tick *Ornithodoros moubata* and harvesting after subsequent mouse passage (type 2). Inocula of *Borrelia* were obtained by diluting infected mouse blood with citrated glucose saline (0.15M saline, 1% glucose, 0.5% citrate with Sorensens buffer to pH = 7.2). Spirochaetes were counted in a Helber counting chamber (Southee, 1972).

Serology. Slide agglutination was tested on a suspension containing 10^8 fresh *Borrelia* in citrated saline. Borreliolysins were also assayed. Assessment was microscopical. Titres were established by determining the highest dilution of antisera at which lysis or agglutination occurred. Borrelial immunofluorescence was tested with goat anti-mouse IgM conjugates (Meloy Laboratories Inc., Springfield, USA) as used (Wright and Ginger, 1973). Antisera were prepared against *Borrelia* from the first, parental (P) and relapse, filial-one (F_1) parasitaemias on both the STIB 503 type 1 and type 2 strains by inoculating pure isolates into mice and collecting sera after recovery from infection had taken place. These specific sera were used in the agglutination and borreliolysin tests.

Mice. All mice used in this study were male or female, 5 to 6 weeks old, 12–15 g weight, inbred CBA/CA strain fed on water ad libitum and on an antibiotic-free diet. When venesection was required, bleeding from the retro-orbital veins or exsanguination by cardiac puncture from the anaesthetised mouse was performed (Wright et al., 1974).

Experiments and results

1. *Tolerance of Borrelia to cyclophosphamide.* Borrelicidal level of cyclophosphamide (Wyeth Brothers, Maidenhead, Bucks.) was determined by giving intraperitoneally 1,000 mg/kg and 150 mg/kg respectively to two groups each of 12 mice. The mice were then exsanguinated by cardiac puncture 75 min later and the sera from 4 mice were pooled. Thus the active cytotoxic alkylating metabolite of cyclophosphamide was obtained (Sladek, 1973). 0.2 ml aliquots of the fresh pooled serum was added to a monolayer of human embryonic lung cells (Flow Laboratories, Scotland). The monolayers were suspended in Eagles minimum essential medium (Flow Laboratories) in 150×15 millimetre Pyrex glass tubing (Corning Glass Works, N.Y. 148390) and sealed by a siliconed bung. A cytopathic effect was sought after 48-h incubation at 37° C. Cytopathic effects were found after 24 h with a sixfold dilution of sera from the high dose cyclophosphamide regime, but not after the low dose regime (Table 1). Equal volumes of fresh serum were also added to spirochaetal suspensions of Wellcome strain of *Borrelia* and incubated for 2 h. The serum had been taken from the cyclophosphamide-treated mice and from the appropriate controls (Table 1). The *Borrelia* were then counted and the serum-*Borrelia* suspension mixture was then inoculated into four mice. The *Borrelia* in the peripheral mouse blood were then counted at 48 h. The "toxic" effect of the sera from mice who had received cyclophosphamide or *Borrelia* paralleled the cytopathic effect in cells

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Table 1. The effect of cyclophosphamide (Cy) on cells and *Borrelia duttoni*

	Cytopathic effect on human embryonic lung	*Borrelia* count/ml	
		2-h incubation in vitro	48 h after infection in vivo**
Borrelial suspension (BS) alone	–	3×10^8	4×10^9
Cy 1,000 mg/kg sera*	+		
plus BS		2×10^7	2×10^7
Cy 1,000 mg/kg sera diluted ⅓	+		
plus BS		2×10^7	6×10^8
Cy 1,000 mg/kg sera* diluted ⅙	+		
plus BS		4×10^8	9×10^8
Cy 1,000 mg/kg sera* diluted $\frac{1}{10}$	–		
plus BS		5×10^8	5×10^9
Cy 1,000 mg/kg sera* diluted $\frac{1}{100}$	–		
plus BS		6×10^8	6×10^9
Cy 150 mg/kg sera*	–		
plus BS		3×10^8	8×10^9
Normal mouse sera*	–		
plus BS		4×10^8	6×10^9
Krebs saline	–		
plus BS		3×10^8	7×10^9
Human embryonic lung cells	–		

  * All sera taken 75 min after giving cyclophosphamide.
** Mean of blood samples from 4 mice.

(Table 1). Incubation of a solution containing 40 mg of cyclophosphamide in Krebs saline with human embryonic lung cells or *Borrelia* produced no cytopathic or borrelicidal effect. The Wellcome mouse strain was uniformly fatal to mice and therefore not used in the following experiments.

    2. *The effect on murine borrelial infection of antibody suppression by cyclophosphamide.* Twelve mice were given cyclophosphamide 150 mg/kg intraperitoneally then infected intraperitoneally with a 0.5 ml of a *Borrelia duttoni* (STIB 503 type 1) suspension of 10^7 organisms per ml. A further 4 mice were given normal saline, the diluent for cyclophosphamide, and then infected with *Borrelia duttoni* as above. Eight further mice were just infected without a preliminary injection. The first parasitaemia (P) was detected 4 days later. The mean number of organisms found in the parasitaemia was 5.4×10^9 borrelia per ml in the animals given cyclophosphamide and 2×10^9/ml in the non-immunosuppressed animals. Eight out of 12 immunosuppressed animals succumbed to the infection, 1 out of 4 mice given Krebs saline and then infected and 4 out of 8 of

Table 2. Serological differences between the first (P) and second (F_1) borrelial parasitaemia

Source of *Borrelia*	Test sera			
	4 days post infection	Anti-P	11 days post infection (anti P + F_1)	Anti-F_1
P control	+	+	+	−
P cyclophosphamide	+	+	+	−
F_1 control	−	−	+	+
F_1 cyclophosphamide	−	−	+	+

4 and 11 day post-infection sera tested for lysins and agglutinins. Anti-P and anti-F_1 tested for agglutinins only.

the infected controls. The second parasitaemia (F_1) was detected 4 days later in the control animals and 5 days later in the immunosuppressed mice. In this second parasitaemia (F_1) the maximal number of spirochaetes seen was 0.5–1×10^6 organisms per ml. None of these animals died. No specific antibodies were detected either against the P or F_1 parasites in sera taken between parasitaemias or 11 days after the initial infection in the immunologically compromised mice. In contrast, antibody in the control mice appeared against the P spirochaetes on the 4th day and against the F_1 parasites on the 11th day following infection. Prior to infection all mice had a negative serology. Antibody titres were: lytic antibody $\frac{1}{10}$–$\frac{1}{40}$, agglutinating antibody $\frac{1}{40}$–$\frac{1}{320}$ and immunofluorescent antibody $\frac{1}{100}$ to $> \frac{1}{500}$. P spirochaetes in both the cyclophosphamide-treated and control animals were found to be serologically identical as was the F_1 variant found in the second parasitaemia (Table 2).

The experiment was repeated using an identical protocol with *B. duttoni* (STIB type 2) strain for producing the experimental infection, i.e. the spirochaete that had been mouse to tick and then mouse passaged. The infecting dose, given intraperitoneally, was 0.2 ml. The first parasitaemia developed on the fifth day. 6 out of the 12 immunologically suppressed mice died and 7 out of the 12 controls. The P variant was found in both groups of mice. No F_1 parasitaemia was detected although by day 11 antibodies against the F_1 strain had appeared in the normal mice. The P parasitaemia again resolved and by the 11th day no antibodies were detected in the mice given cyclophosphamide.

Discussion

The mouse can recover from borrelial infection without measurable specific antibody being demonstrated. More surprisingly, antigenic variation may occur as seen in the initial mouse experiment. Although no borrelial antibody was detected in the cyclophosphamide treated mice, it is possible that antibo-

dies inducing antigenic variation could still develop. Cyclophosphamide may enhance this type of antibody production, perhaps by inhibiting suppressor thymus derived lymphocytes (Thomas et al., 1977). If production of antibody modulating antigen was thereby enhanced, this antibody would not only protect against infection, but would induce antigenic change in the immune compromised mice. The failure in mice to demonstrate delayed hypersensitivity to spirochaetes or to find haptenic cardiolipin antibodies in *T. pallidum* infected mice, which would presumably require T-lymphocyte help for production (Wright et al., 1974), does not necessarily exclude T-lymphocyte suppressant activity. The deletion of these cells may therefore allow increased production of the antibody which modulates the variation.

The possibility that there might be two antigenic variants of *Borrelia* inoculated simultaneously in the above experiments is not likely as the findings do not account for the "spontaneous" cure or explain why the F_1 variant could be passaged without P spirochaete antibody being produced. Alternatively it might be suggested that lytic antibody was produced in such small quantities that it was "complexed" with the circulating antigen, lysed the bacteria leaving no antibody to be detected (Schuhardt and Wilkerson, 1951). The inability to detect antibody in the immunosuppressed animals even after the second parasitaemia makes this suggestion unlikely.

The activated cyclophosphamide metabolites are unlikely to induce the antigenic change, as the same variants appeared whether or not the mice had received cyclophosphamide. Activated cyclophosphamide is of transient duration in the mouse (Berenbaum et al., 1973) and in the concentration of cyclophosphamide used in the animal experiments, showed no inhibition of in vivo growth of the mouse adapted strain of spirochaetes. Antigenic variation may be dependent on antibody, but still environmentally influenced. Paramecium undergo antigenic changes with differences in environmental temperature and salinity (Beale, 1974). The occurrence of cross agglutinating antibody in alternate relapses reported by Cunningham et al. (1934) does not rule out the possibility that antigenic variation may be an inherent, perhaps a genetically controlled characteristic.

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