ANTIGENIC VARIATION OF *BORRELIA HERMSII*

By HERBERT G. STOENNER,* THOMAS DODD, and CAROLE LARSEN

From the Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840

Since the discovery of *Borrelia recurrentis* as a cause of relapsing fever (1), biologists have been intrigued by the ability of the organism to cause repeated attacks of disease in man. Early investigators attempted to elucidate the relapse phenomenon by comparing the serologic responses of recovered animals with original and relapse populations of spirochetes. Most found that peak antibody titers to relapse spirochetes subsequently appeared in the order in which the spirochete populations were isolated (2). This finding suggested that the surface antigens of the organism had been changed and that growth of a modified population was responsible for each relapse. However, this concept was not uniformly accepted. Some investigators could not detect an immune response with any serologic test then in use. As explained in a review by Schuhardt (3), there were many reasons for controversy about early theories on the mechanisms of relapse. Many strains studied had been grown by repeated passage in animals without a complete record of factors that affected the relapse phenomenon. A variety of tests were used to measure serologic responses, and there was serious disagreement about which tests gave valid results. In addition, the phenomenon was different in the many animal hosts used to study the process.

In recent years, little progress has been made in elucidating the true nature of the phenomenon. According to one popular concept (3, 4), new serotypes arise from borreliae that escape the specific antibody response and retreat into organs where new antigens are unmasked. These changed organisms penetrate the blood stream and cause a relapse that, in turn, is terminated when the host produces a specific serologic response to that relapse population. This process is repeated until death intervenes or a complete immunity is established.

The development of a medium for cultivating borreliae (5, 6) and the demonstration by Coffey and Eveland (7, 8) that individual organisms in a relapse population can be identified by specific fluoresceinated antiserum could have enabled new approaches to a study of the phenomenon. Investigating the relapse phenomenon in rats, they identified four serotypes of *B. hermsii* with rabbit and rat antisera conjugated with fluorescein-isothiocyanate (FITC)[1] according to a modification of the method of Gordon et al. (9). Initially, we attempted to repeat their work with antisera prepared in rabbits against the original and three relapse populations of *B. hermsii* obtained from a single mouse. Borreliae in smears of mouse blood were stained with these antisera by indirect immunofluorescence, but organisms from one population could not be clearly distinguished from those of the other three. Furthermore, the variability

---

* Retired from the U. S. Public Health Service. Current address: 1102 S. 2nd, Hamilton, MT 59840.

[1] *Abbreviations used in this paper:* FITC, fluorescein-isothiocyanate; PBS, phosphate-buffered saline; PI, postinoculation; RML, Rocky Mountain Laboratories.

in brilliance among individual organisms suggested that the original and the three relapse populations contained mixtures of serotypes. From preliminary studies made with cloned populations, it became apparent that the relapse phenomenon was extremely complex, and it involved many more than four serotypes identified by Coffey and Eveland (7, 8). In this report, the procedures for isolating and identifying serotypes and observations on the dynamics of seroconversion are described.

Materials and Methods

B. hermsii. The HSI strain of *B. hermsii* used was isolated from *Ornithodoros hermsi* collected near Spokane, WA by Dr. Willy Burgdorfer (Rocky Mountain Laboratories [RML]), (10).

Mice. Most experiments involving animals were conducted on 18–19-d-old Swiss mice of the RML stock. Mature female mice, 6–7-mo old (nonproductive breeding stock), were used for preparing antisera to various serotypes. BALB/c *nu/nu* mice of the RML substrain were used in experiments conducted to evaluate the role of antibody on conversion of serotypes. All mice were inoculated intraperitoneally.

Cloning of B. hermsii and Preparation of Pools of Live Antigens. After it became obvious that relapse populations initially studied contained mixtures of serotypes, a cloned population was established from a mouse that had been fed upon by an infected *O. hermsi* tick. The organisms in freshly drawn citrated mouse plasma were counted (11), and then 12 mice were each inoculated intraperitoneally with 0.1 ml of a suspension containing five organisms per ml. Mice were usually inoculated at 4:00 p.m. on a given day, and then their blood was examined for spirochetes 88–90 h later. For this examination, a small amount of blood, obtained by tail clip, was placed on a glass slide adjacent to an oval deposit of Kelly's medium (5). As the tail touched the slide, it was moved toward the medium until the blood moved into the edge of the medium. When the slide was placed in a warm dark-field condenser, the drop of blood would spread near the surface of the medium. Spirochetes were readily visible near the spreading edge of the blood and between clumps of erythrocytes. The minimum level of spirochetemia detectable by this method was ~3 × 10^3 organisms/ml. The three mice that developed the greatest spirochetemia were bled, and glycerol was added to a 10% concentration to the individually citrated plasma of two mice and frozen by submerging vials in alcohol at -68°C. Throughout these experiments, phosphate-buffered saline M/15, pH 7.3 (PBS) was added to equal 50% of the volume of blood taken by cardiac puncture, to increase the yield of borreliae in the plasma after centrifugation to remove blood cells. Starting with plasma of the third mouse, we made 3–4 passages at 36–48-h intervals in increasing numbers of mice to obtain sufficient stocks of live organisms. Plasma of mice with the greatest spirochetemia was used for each passage. Passages in which a marked increase in spirochetemia was not achieved were excluded. Stocks of organisms made after the first four batches were prepared in mice given 300 mg of cyclophosphamide/kg body weight (Cytoxan, Mead Johnson & Co., Evansville, IN) 1–2 h before inoculation with borreliae.

Search for Serotypes and Preparation of Conjugated Antisera. In an initial attempt to recover new serotypes, we inoculated six mice with ~10^5 organisms of the first cloned population (type 1), and the infection was monitored by dark-field microscopy. Relapse populations from this experiment and others were compared with each other by reciprocally staining organisms with their antisera. Each relapse population was cloned before stocks of organisms were prepared for immunization of mice. 12 female mice, 6–7 mo old, were each inoculated with ~2 × 10^6 borreliae. After 36–40 h post-inoculation (PI), these mice had access only to drinking water containing 600 mg of chlortetracycline hydrochloride/liter of water. This treatment prevented growth of variants in the blood stream, which could arise from the population sequestered in the brain. Beginning on the 6th d after inoculation and repeated four times at 3-d intervals thereafter, each mouse received a booster dose of ~2 × 10^6–10^7 organisms of frozen stock. Most organisms were viable. 6 d after the last booster dose, mice were bled by cardiac puncture. As mice were bled, PBS was added to equal 50% of the volume of blood obtained from each mouse. The resulting serum was considered to be a 1:2 dilution.

Except for the following modifications, the procedure of Peacock et al. (12) was used for conjugating antisera. Because a 1:2 dilution of mouse serum was conjugated, the quantity of

reagents prescribed was reduced by one-half. Also, FITC was allowed to couple with immunoglobulins during incubation for 2 h at 35°C, instead of 45 min at 37°C. Conjugated antisera and working dilutions thereof were stabilized with bovine albumin added to a 1% concentration (13). Reserve stock conjugated antiserums were kept frozen at −68°C, and working quantities of undiluted and diluted sera were held at 4°C. Antisera were diluted so that homologous organisms stained brilliantly and heterologous organisms stained faintly (see Fig. 1). This staining procedure enabled calculation of the percentage of homologous and heterologous organisms stained by each antiserum. If none of the organisms stained brilliantly, the population was considered to represent a serotype(s) other than those contained in the battery of antisera used in a given test. Because working dilutions gradually lost activity when held at 4°C or when exposed to room temperature, their concentration was periodically adjusted to achieve proper staining.

Serotyping Borreliae in Blood of Mice.   Spirochete populations in mice were characterized according to serotype by the procedure outlined (Table I). For the preparation of good slides, it is important that sufficient blood cells are taken into a capillary pipette and mixed with the plasma, so that, when the mixture is spread, ~25–50% of the glass slide will be covered with a single layer of erythrocytes.

Media.   During the early part of the study, borreliae were cultivated in modified Kelly's medium (11) and suspended in this medium during cloning procedures. After we discovered that serotypes grown in this medium rapidly lost ability to express their characteristic surface antigens and that the viability of small inocula was not well protected, further modifications were made by adding CMRL tissue culture medium to a 5% concentration (330-1540; without glutamine and sodium bicarbonate, Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). This supplement was evaluated because it had been used for cultivating *Spiroplasma mirum,* a fastidious organism (14). Also, yeastolate (Difco Laboratories, Detroit, MI) was added to a 0.2% concentration (6) to the complex medium hereafter termed fortified Kelly's medium. It should be noted that CMRL medium gradually lost its ability to enhance growth as the shelf-life expiration date neared (6 mo). Because a precipitate formed when these supplements were mixed with Kelly's basal medium, the final medium was filtered through a coarse filter before it was used to count borreliae for cloning.

In the interest of clarity, some special procedures used in experiments concerning the dynamics of antigenic variation are given in the Results section with the experiments concerned.

Results

Establishing Battery of Serotype Antigens and FITC-conjugated Antisera.   The first three relapse populations of one mouse were cloned and then compared with the original and with each other by reciprocally staining blood smears of each with their four FITC-conjugated antisera. By the foregoing procedure, we showed that this mouse had been infected with at least four serotypes. By similarly examining the remaining relapse populations from the first experiment, six were shown to be types 1, 2, 3, or 4 or mixtures thereof, but five contained populations other than the serotypes so far identified. These five were then cloned, FITC-conjugated antisera were prepared, and each was compared with the rest and shown to be different. As new serotypes were identified, antisera were prepared against them, and their relapse populations were studied for additional new serotypes. The search was discontinued when 24 serotypes were identified.

With the battery of 24 specific antisera, we then determined the order in which conversions occurred and whether a variant would regain its original surface antigens through a series of conversions. In this experiment and in all subsequent experiments involving defined inocula, counts were made of borreliae derived from freshly-drawn citrated blood of mice taken 24–44 h after inoculation. This time limit was selected to preclude any significant antigenic shift of borreliae used for inocula. After mice were
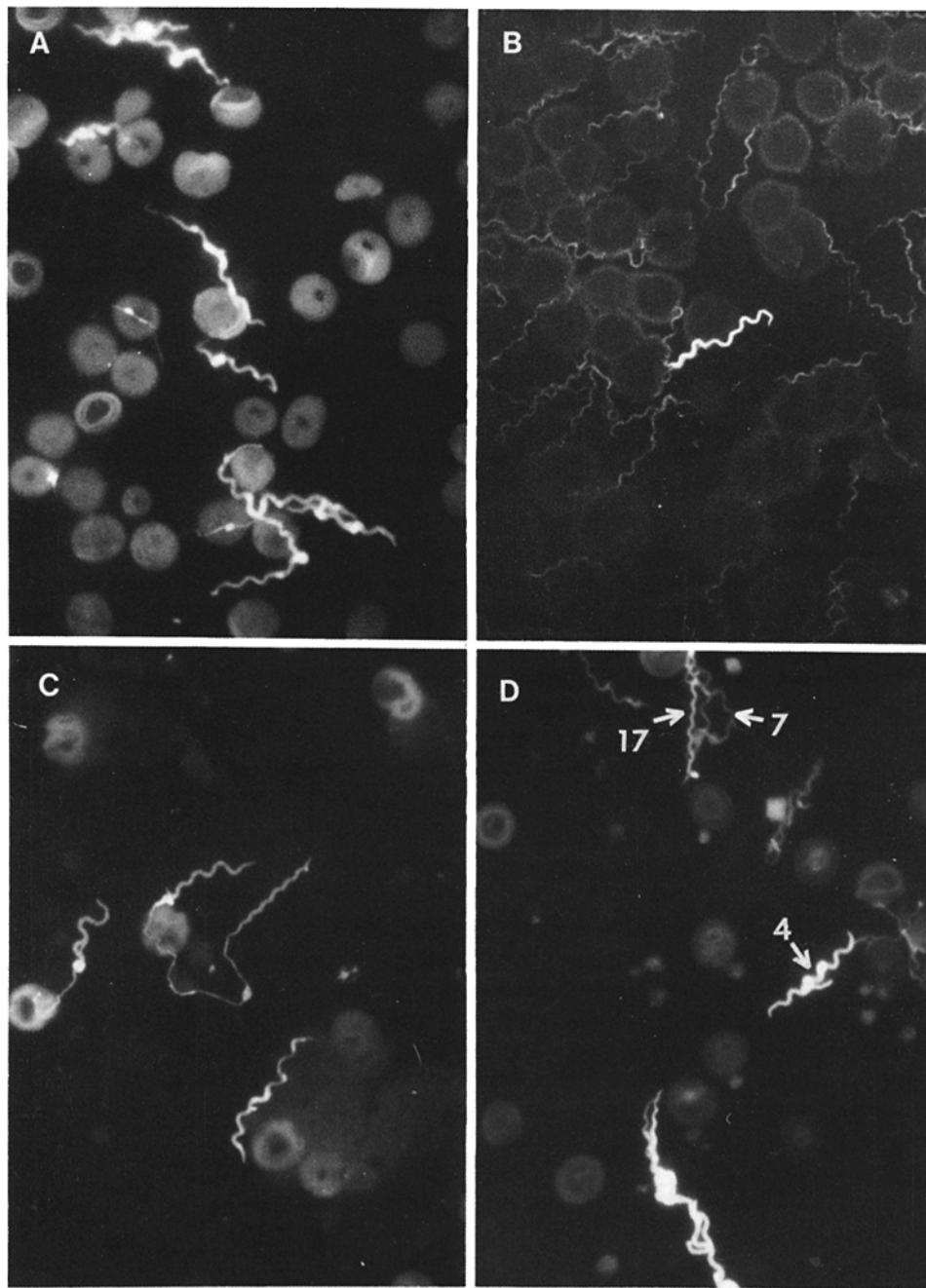
FIG. 1.   (A) Pure population of type 7 stained with its homologous antibody. (B) Type 3 contaminant in an otherwise pure type 7 population stained with type 3 antibody. (C) Homologously stained type 21, connected with a thread-like extension. (D) Mixed population of types 4, 7, and 17 stained with type 4 antibody. Type 17 cross-reacts with type 4 antibody.

TABLE I

Procedure for Serotyping Borreliae in Blood of Mice

Clip tail of mouse, beginning 48 h PI and examine blood by dark-field	
A	*B*
1. If 10^6 borreliae/ml estimated, obtain citrated blood by cardiac puncture.	If insufficient number at 48 h, repeat dark-field examination at 72 h.
2. Centrifuge blood, prepare slide of plasma, and fix in absolute methanol for 30 min at 35°C.	If 10^6 borreliae/ml, follow A. If borreliae are present, but still insufficient, transfer 0.1 blood to amplifier mouse.
3. Stain with FITC-conjugated antisera for 30 min at 35°C.	
4. Wash slide in PBS for 7–8 min.	Clip tail of amplifier mouse at 48 h PI and examine
5. Read slides under Orthoplan microscope (E. Leitz, Inc., Rockleigh, NJ).	blood by dark-field. If 10^6 borreliae/ml, follow A. If insufficient, repeat dark-field examination at 72 h, then follow A.

inoculated with 25 organisms, borreliae were regularly seen in their blood on the 3rd and 4th d PI (and sometimes on the 5th), and clearance of the original population was usually precipitous. During preliminary studies we showed that the first antigenic change was associated with clearance of the original population from the blood and was not related to the occurrence of relapse. Therefore, on the 6th and 7th d PI, 0.1 ml of blood was transferred from each of the 12 test mice to a normal mouse to amplify the population for characterization by fluorescent antibody (FA) tests (Table I). Blood from mice with no detectable spirochetemia was also passed to amplifier mice. In a few instances, the type inoculated into the test mouse persisted on the 6th d, but generally the population present on the 6th and 7th d were first relapse organisms.

Staining Properties and Serologic Relationships. Each serotype was stained specifically by its homologous antibody, and most were readily distinguished from the other 23 (Fig. 1). Homologously stained organisms appeared thicker in diameter and frequently contained brilliantly stained knobs or enlargements. Some had what appeared to be long extensions of axial filaments, perhaps one-quarter the diameter of the rest of the organisms. These extensions were frequently seen in smears of cultures. In dark-field preparations, two separate organisms joined by these faintly visible extensions were found to move simultaneously in the same direction. Sometimes the specifically stained component of the organism would be spread over an area 15–30 μm Diam, with the skeletal remains of the organism in its midst. A few cross-reactions caused some initial difficulty in identifying serotypes until they were recognized and evaluated by studying mixtures of the two organisms concerned. These cross-reactions were typically 2 plus in brilliance, compared with a 5 plus homologous reaction (Table II, Fig. 1). Generally, cross-reactions were more pronounced in organisms situated near the periphery of the drop of antiserum, and less prominent in smears that had too many erythrocytes. A troublesome one was a serotype that commonly emerged from type 7 (Tables II and III) and consequently was immunologically different. Serologically, this type showed extensive cross-reaction with 7 and 16 antibody. Yet no reaction was seen between types 7 and 16 in reciprocal tests of these organisms and their respective antisera. This new serotype was presumed to be present when the same percentage of organisms were moderately stained with 7 and 16 antisera.

Order of Emergence of Serotypes and Frequency of Their Occurrence. On the 6th and 7th d after inoculation with 25 borreliae, 60% of mice had detectable spirochetemia; yet, as

Table II

Serologic Relationships among 24 Serotypes of B. hermsii, as Detected by Reciprocal Fluorescent Antibody Tests

Fluorescent antibody	Organisms																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	5*																							
2		5																						
3			5																					
4				5													2					2		
5					5												2		2					
6						5																		
7							5									(4)‡								
8								5																
9									5	2														
10										5														
11							2				5									2				
12		2							2	2		5												
13			2										5											2
14														5										
15										2	2				5		2							
16							(4)‡									5								2
17																	5							
18																		5						
19						2													5					
20																				5				
21																					5			
22							2															5		
23			2																				5	
24							2										2							5

* Numerals indicate level of reaction. Blank spaces indicate no reaction beyond background staining.
‡ Denotes new serotype that cross-reacts with 7 and 16; 7 and 16 do not cross-react with each other.

judged by blind passage, nearly all mice were spirochetemic. Of a potential of 476 mouse-days of observation (288 mice on days 6 and 7), emerging types were identified on 419 mouse-days; results on other days were invalidated because mice died of cardiac puncture on day 6 or growth of borreliae in amplifier mice was inadequate for typing. There was considerable variation in the number of serotypes that emerged, but many of the less frequent of the longer lists were represented by only one or two isolates. The results of these experiments indicate a wide variation in frequency of occurrence of types (Table III). In order of decreasing frequency, the ten most common serotypes encountered were 7, 2, 17, 24, 13, 3, 1, 21, 11, and 12. During 419 mouse-days on which emerging types were satisfactorily identified, types 7, 2, 13, 3, 21, 4, and 14 were seen 313, 175, 80, 42, 41, 39, and 31 times, respectively. These data suggest that conversions do not occur at random, but are programmed in some manner. Indeed, this thesis was supported by the results of three repeated experiments in which some minor serotypes were regularly dominant among the emerging population of selected ones. Thus, 4 commonly emerged from 15, and 13 from 23 (Table III).

Population Changes During Antigenic Conversion. The next experiment was conducted to establish the population changes associated with conversion. In this experiment, 15–18 mice were each inoculated with one organism of types 7, 3, or 21 and then, beginning on the 4th d PI, 0.15 ml blood was obtained daily by cardiac puncture from each of 12 spirochetemic mice. 0.10 ml was passed to an amplifier mouse, and 0.05 ml was used for counting the organisms (11). If the plasma was relatively free of lipids, populations as low as 800 cells/ml blood could be counted, but in most cases borreliae could not be counted accurately in dilutions below 1:2 (1,600 cells/ml).

TABLE III

Serotypes Emerging During the First Relapse Period (Days 6 and 7) PI in Mice Inoculated with 25 Organisms of a Given Serotype

Serotype inoculated	Origin of serotype	Order of decreasing frequency of emerging serotypes														
1	O. hermsi tick	7	2	17	24	20	14	13	9	1	(2 new)					
2	Exp* 1, M1	7	17	13	22	4	6	20	21	24	9	3	1	14	16	
3	Exp 1, M1	7	13	17	2	11	7 × 16‡	6	24							
4	Exp 1, M1	7	17	2	13	6	1	11	12	4	9	14	15	23	19	
5	Exp 1, M1	7	2	20	24	11	13	19	1	6	14	17	18	21	(2 new)	
6	Type 1	7	17	2	24	3	1	16	21	(1 new)						
7	Type 3	21	2	7 × 16‡	17	24	11	1	6	20	19	18	14	(2 new)		
8	Exp 1, M3	7	17	24	21	13	3	22	1	2	20	18	19	14	15	6 (4 new)
9	Exp 1, M5	2	7	24	12	1	21	14	16							
10	Exp 1, M6	7	24	2	17	21	1	3	10	12	9	13	16	18		
11	Exp 1, M2	7	24	17	2	11	13	20	21	14	8	2	3	(4 new)		
12	Exp 1, M3	7	17	24	2	10	14	16								
13	Type 4	3	17	7	2	4	21	22	24	1	8	14	16	18	20	(1 new)
14	Type 7	17	7	12	24	4	2	10	7 × 16‡	20	11	1	21	9	3	(3 new)
15	Type 8	4	7	2	23	13	14	3	20	21	18	17	16	6	9	19 8 (4 new)
16	Type 7	7	24	2	20	17	11	16	6	14						
17	Type 9	7	2	13	11	6	15	17	12	14	1	4	16	18	21	24
18	Type 6	17	13	7	15	12	8	3	2	1	(1 new)					
19	Type 5	7	2	13	6	1	5	11	17	24	3	4	19	21	(1 new)	
20	Type 1	7	17	24	14	2	3	21	13	(10 new)						
21	Type 17	7	2	12	17	7 × 16‡	1	9	11	8	13	14	(1 new)§			
22	Type 15	7	17	2	21	18	14	3	8	13	1	16	6	9	20	24 (1 new)
23	Type 4	13	7	4	2	24	17	12	16	20	1	6	9	14	10	19
24	Type 5	7	8	17	2	3	13	16	6	9	20	(5 new)				

* Experiment.

‡ New serotype that cross-reacts strongly with 7 and 16.

§ Emerging serotypes not tested against type 24 antiserum.

Only representative findings are presented (Table IV) because data are too voluminous and population changes of some mice were not completely defined, as some mice died from repeated cardiac puncture.

After inoculating mice with a single organism (or statistically, very few) (15), most had developed spirochetemia on the 4th d ranging from 10,000 to 800,000 borreliae/ ml (Table IV). Infections in mice with low counts usually had not reached a peak by the 4th d, and the number of organisms increased until the 5th d. On the 4th d PI, most mice had a pure population of the inoculated serotype, but, in some, conversion had begun; a few mice still had a pure population on the 5th d. A prominent feature of the initial conversion was a sudden clearance of an apparently pure population, from which as many as seven serotypes emerged the following day. In a special effort to detect small numbers of emerged types among organisms during peak spirochetemia, we examined as many as 3,000 borreliae in many amplifier mice, but emerging types were rarely found. Thus, it appeared that only a small fraction of the population at any given time converted, and the event was coordinated with destruction of the initial population, presumably by antibody produced by the host. Note that emerging serotypes generally did not persist in the blood of mice for >2 d, if a significant population of that serotype developed during that period.

Mechanisms of Conversion.   Two hypotheses about the mechanism of conversion were considered in planning further experiments. Because of the sudden change from a pure population to a mixture of as many as seven serotypes (Table IV), it appeared that the event may be induced by early antibody production; the alternative hypoth-

TABLE IV

Population Changes among Serotypes of B. hermsii Undergoing Conversion in Mice Inoculated with One Organism of a Given Serotype

Mouse	Serotype inoculated	Number of borreliae of indicated serotypes/ml blood on day PI				
		4th	5th	6th	7th	8th
J1-m1	7	7(240,000)	1(800), 2(20), 6(200), 7 × 16*(200), 24(800)	1(22,800), 2(7,600), 6(3,800), 7 × 16(7,600), 13(15,200), 17(3,800), 24(7,600)	2(160,000), 7 × 16(320,000), 17(16,000), 24(1,280,000)	14(<80), 17(<720)
J1-m3	7	7(180,000)	1(100), 2(200), 6(600), 7 × 16(1,200)	7 × 16(6,000), 24(6,000)	21(7,000), 24(133,000)	2(40), 9(40), 12(200), 17(3,600)
J2-m2	7	7(60,000)	7(840,000)	7 × 16(8,000)	2(40,000), 7 × 16(44,000), 21(4,400)	New (8,000)
J3-m3	7	7(720,000)	7(800,000)	2(<2,000), 7 × 16(<1,600), 21(<200), 24(<200)	2(2,000), 7 × 16(8,000), 14(2,000), 17(8,000), 21(2,000)	14(400), 17(3,600), 21(200)
J1-m2	3	3(128,000)	3(520,000)	2(<240), 7 × 16(<320), 24(<240)	7 × 16(<1,600)	7(14,000)
J1-m3	3	3(440,000)	1(7,200), 2(7,200), 3(706,600)	2(24), 7 × 16(1,480), 24(720)	7 × 16(<1,600)	7 × 16(1,600)
J2-m2	3	3(56,000)	2(400), new (3,600)	2(6,600), 7(6,600), 20(12,000), 24(105,600)	20(35,000)	17(4,800)
J1-m1	21	7 × 16(20,000), 21(380,000)	7 × 16(16,000)	2(480), 12(320)	2(100,000), 12(100,000)	5(640), New (120)
J1-m4	21	21(360,000)	7 × 16(<1,600)	1(<16), 2(<40), 6(<80), 7 × 16(<240), 12(<240), 14(<40), 17(<40)	6(600), 7 × 16(8,400), 12(8,400), 14(600), 17(240), 24(600)	7 × 16(800)
J2-m5	21	21(16,000)	21(400,000)	7 × 16(160), 12(320), 24(960)	7 × 16(14,000), 12(6,000)	7 × 16(22,000), 12(6,600), 17(15,400)

* New serotype that cross-reacts strongly with 7 and 16.

esis was that the event occurred spontaneously, independent of antibody production. Because small numbers of new serotypes could be present but obscured at peak spirochetemia, it became necessary to determine the generation time before the shift in population could be evaluated. For serotype 7, whose growth rate is representative of many others, the generation time was ~3 h (2.8–4.2) during logarithmic growth in naïve mice (16). Thus, in most cases (Table IV), it would be impossible to detect small numbers of newly emerged serotypes among an apparently pure population at peak spirochetemia. For example (type 7, J1-ml), only 3 type 1 organisms are needed on day 4 to account for 800 on day 5, and it is impossible to detect that few among 240,000 type 7. However, a number of exceptions were noted (not all shown). For example, one should have been able to detect 156 type 2 organisms among 8,000 (type 7 × 16 third line J2-M2) on day 6 that would be needed to account for 40,000 on day 7. Hence, further evaluation of the role of antibody in conversion appeared necessary.

Role of Antibody Production in Induction of Conversion of Borreliae. This question could be resolved readily by comparing the conversion rate of borreliae in nude mice with that in normal mice, if antibody production to antigens of borreliae was thymus dependent. However, conversion did occur in nude mice inoculated with one type 7 organism; variants appeared at the same time in nude mice, but peak spirochetemia

was 10-fold lower than in RML mice. Because this approach failed, alteration of the immune response by chemical suppression was attempted.

In preliminary studies (H. Stoenner, unpublished observations), we showed that cyclophosphamide (300 mg/kg) would usually delay clearance of the initial population by ~36 h. If early antibody production directly influenced conversion, the number of variants in a population just before clearance of organisms in the controls should be much lower in cyclophosphamide-treated mice (17). However, to isolate and identify variant organisms in either group, it would be necessary to eliminate the dominant population. For this purpose, antibody specific to serotype 7 was prepared by inoculating adult mice with ~5 × 10^6 organisms and treating each of them 48 h later with 25,000 U of penicillin G per mouse, given intraperitoneally (sodium salt of benzyl penicillin). 3 d later mice were bled and serums were pooled and filtered through a 0.2-μm filter to remove any borreliae that may have survived penicillin treatment.

Cyclophosphamide (300 mg/kg) was administered intraperitoneally to eight mice, and 2 h later, these and eight control mice were each inoculated with one type 7 organism (Table V). About 100 h later, the organisms in plasma of 10 mice with the greatest spriochetemia (5 principal and 5 control) were counted and varying numbers

TABLE V

Effect of Immunosuppression of Mice on Conversion of Type 7 B. hermsii*

Mouse number	Organisms/ ml plasma	Variants that grew in amplifier mice inoculated with the following doses:			
		100,000	10,000	1,000	333
Cyclophosphamide-treated					
1	6.8 × 10^6	24, 7 × 16‡	24, 7 × 16	24	0
2	8 × 10^4	ND§	24	0	0
3	1 × 10^7	24, 2, 20, 17, 7 × 16	14	24, 2	0
4	4 × 10^6	20, 7 × 16, 2	20, 17, 6, 7 × 16	20, 2, 7 × 16	0
5	1.8 × 10^5	ND	24	24	0
6	8.4 × 10^6	24, 20, 2, 7 × 16	24	0	0
7	8 × 10^5	24, 17, 7 × 16	0	0	0
8	8.6 × 10^6	24, 2	24, 2	0	0
9	7.2 × 10^6	24	24	0	0
10	1 × 10^6	2, 24, 7 × 16	0?‖	2	0
Controls					
1	3.6 × 10^6	20, 24, 6, 17, 2	24, 2, 7 × 16	0	0
2	4.8 × 10^6	2, 24	0	0	0
3	1.0 × 10^7	6, 20, 24, 2, 7 × 16, 14	7 × 16	7 × 16	0
4	3.2 × 10^6	24, 6	24	24	0
5	2.2 × 10^6	2, 20, 24, 7 × 16, 14	0	0	0
6	1.2 × 10^6	2, 24	24	0	0
7	1.5 × 10^5	ND	24	0	0
8	1.5 × 10^6	24	24	0?	0
9	2.4 × 10^6	2, 6, 21	2	0?	0?
10	1.5 × 10^6	24, 17, New	24, New	0	0

* Mice were treated with 300 mg cyclophosphamide/kg 2 h before inoculation with one type 7 organism.
‡ New serotype that cross-reacts with 7 and 16.
§ Not done.
‖ Results questionable. Antiserum insufficient to control growth of type 7.

were inoculated intraperitoneally into mice that had been treated ~1 h before with 0.2 ml of a 1:16 dilution of 5-d anti-7 serum given by the same route. The blood of test mice was examined on the 3rd and 4th d PI. Passage of 0.1 ml whole blood was made to amplifier mice on the first day of spirochetemia. Blood from all the rest of the mice was passed on the 4th d, regardless of spirochetemia. This experiment was repeated, and the results of both are compiled (Table V). The concentration of new serotypes varied among mice, but the number and types in cyclophosphamide-treated mice did not differ significantly from those in control mice. Thus, it appeared that early antibody did not markedly influence conversion.

Spontaneous Conversion in Cultures.   Next, we tested the hypothesis that conversion occurs spontaneously, independent of antibody. If it occurred in this manner, variants should appear in cultures of Kelly's medium inoculated with a single serotype. It would have been ideal to initiate growth from a single organism, but unfortunately, ~800 were necessary to achieve growth (11). Nevertheless, some preliminary studies were made of types 1, 3, 7, and 14 in Kelly's medium inoculated with mouse blood containing ~10^6 organisms/inoculum. During the first culture passage, most of the organisms of each serotype had lost ability to express their characteristic surface antigen. A fluoresceinated antiserum was prepared against a cloned population of the changed organism from type 7, and we were surprised to learn that most organisms in cultures of these four serotypes now expressed a common antigen termed "culture type." When these mixed cultures were inoculated into mice, the culture type persisted for two to three mouse passages, but it was eventually replaced by the original serotype inoculated into the culture.

Because plain Kelly's medium was not useful in resolving the question, an attempt was made to improve the medium. As CMRL tissue culture medium had been used to enhance growth of mycoplasma, a fragile organism (14), we attempted to cultivate borreliae in this medium, but failed. However, when it was added to a 5% concentration to Kelly's medium already fortified with 0.2% yeastolate (6, 11), rapid growth was regularly obtained in 8.5-ml culture tubes inoculated with a single organism. Furthermore, most of the organisms expressed their characteristic surface antigen during the first culture passage.

Tubes of fortified Kelly's medium were then inoculated with a single organism of type 7 obtained from freshly drawn mouse blood. Cultures were held at 35°C and monitored for growth, beginning ~13 d after inoculation. When it was estimated that maximum growth had been achieved, the population in each tube was counted and mice were inoculated with varying numbers of borreliae. About 1 h before inoculation with organisms, mice were treated with 0.2 ml of a serum mixture of a 1:16 dilution of anti-7 and a 1:64 dilution of anti-culture-type serum (unconjugated serum from hyperimmunized mice). This serum mixture, given intraperitoneally, prevented infection by the dominant 7 and any small numbers of culture type in the inocula. A 5-ml portion of each culture was centrifuged to sediment organisms, the supernatant medium removed by aspiration, and the sediment resuspended in 0.5 ml of mouse blood diluted 1:5 in PBS. Slides of these suspensions were stained with fluoresceinated antisera and examined for variant serotypes. A similarly designed experiment was conducted with type 21.

The occurrence of variants in cultures originating from a single organism is proof of spontaneous conversion (Tables VI and VII). The concentration of variants

TABLE VI

Spontaneous Conversion of B. hermsii, Type 7, in Tubes of Fortified Kelly's Medium Inoculated with One Organism

Tube number	Organisms/ml at harvest	Variants seen in smears of cultures	Variants that grew in mice* inoculated with the following doses:			
			100,000	10,000	1,000	333
1	1.36×10^6	24, 18, 17‡	17, 24, 2‡	24	24	0
2	1.72×10^7	24, 20, 6	New, 2	0	0	0
3	7.6×10^6	24	24, 20	24	24, 20	0
4	1×10^7	0	2, 6, 14, 20, 24	2, 24	24	0
5	1.48×10^7	24	20, 24, 6	20, 24	24	0
6	5.2×10^6	24, 6, 18	20, 24, 17, 2	17, 24	24, 20	24
7	4×10^6	24	2, 6, 14, 17, 20, 24	20, 24	20, 24	0
8	2×10^7	24, 17	24, 20, 1, 17, 21	0	0	0

* Before inoculation, mice were treated with 0.2 ml of mixed immune serum to type 7 (1:16 dilution) and to culture type (1:64 dilution), given intraperitoneally.

‡ Listed in order of decreasing frequency.

TABLE VII

Spontaneous Conversion of B. hermsii, Type 21, in Tubes of Fortified Kelly's Medium Inoculated with One Organism

Tube number	Organisms/ml at harvest	Variants seen in smears of cultures	Variants that grew in mice* inoculated with the following doses:			
			100,000	10,000	1,000	333
1	4×10^7	24, 17, 12, 20, 2, 7‡	12, 24, 17, 7‡	IGFA§	24, 17, 12	24
2	2.4×10^7	24, 7, 12, 17	24, 17, 12, 7	12, 24, 17, 2	12	0
3	2.4×10^7	24, 7, 12, 9, 17	12, 17, 24	7, 7 × 16, 24, 2	7, 12	0
4	3.2×10^7	24, 12, 14, 7, 17, 2	24, 12, 17, 7, 14	12, 24, 17, 14, 7	12, 24, 7	0
5	1×10^7	12, 24, 2	24, 17, 12	24, 12, 2, 17, 7, 20	12, 24, 17, 7, 20	0
6	1×10^7	12, 2, 17	12, 17, 24	14	0	0
7	4×10^6	24, 17, 22, 7	7, 12, 2, 7 × 16	12, 7, 14, 17	12, 14, 7, 24	14, 24
8	2.8×10^7	24, 12, 17, 7, 2, 14, 15	12, 7, 7 × 16, 17, 2	12, 17, 14, 24	12, 17, 24	17, 24

* Before inoculation, mice were treated with 0.2 ml of mixed immune serum to type 21 (1:16 dilution) and to culture type (1:64) given intraperitoneally.

‡ Listed in order of decreasing frequency.

§ Insufficient growth for identification by FA staining.

originating from type 7 was roughly comparable to that seen in populations grown in mice (Table V). Furthermore, the variation in concentration is consistent with that expected in a spontaneous change, as the event could occur at any time during logarithmic growth. Tubes in which the event occurred early would contain a higher concentration than those in which the event occurred late in the growth curve. The number and concentration of variants in cultures of type 21 was higher than that seen in cultures of type 7. However, type 21 cultures had more opportunity to convert because the yield of organisms at harvest was greater than that of type 7 cultures. In most instances, variants that appeared in mice were also detected in smears of cultures that had been inoculated into mice. None of the tubes of cultures of type 7 or 21 had >1% culture type at time of harvest (data not shown).

Discussion

Our findings on conversion and its dynamics have clarified much of the long-standing confusion about the pathogenesis and serology of relapsing fever caused by

B. hermsii. It is likely that spirochetemia in man, like that in mice, is persistent, and that relapses are diagnosed when populations reach detectable levels. Although borreliae will persist in immunologically compromised sites like the brain and eye, such sites are not essential for the spontaneous conversion of the organism and ensuing relapses caused by variants. In past research, much confusion arose because investigators assumed that relapse populations consisted of a single serotype. The finding that these relapse populations are frequently mixed explains the contradictory findings of early workers (3), who contended that antibodies to borreliae could be measured by an unusual variety of serologic tests, including agglutination, adhesion, opsonic activity, immobilization, and spirochetolysis tests. Those who contended that none of these tests were of any value were probably right, because the antigenic composition of populations would change during a brief series of passages. Furthermore, the agglutinability of different serotypes varies. As observed in darkfield preparations, type 7 remains uniformly dispersed, whereas others, e.g., types 14, 16, and 21, adhere to each other and blood elements they contact. Organisms of these types are often seen with their tails joined and bounding about the field in the form of a circle.

The mouse antisera used in this study were remarkably specific. This characteristic has been recognized before in the identification of closely related strains of *Rickettsia rickettsii* (18, 19). The specificity of antisera was not marked until mice had received three booster doses of organisms. Hence, they were given two additional boosters to assure adequate differentiation of serotypes. Probably one reason for the serotype specificity of these sera was that mice were immunized with living organisms whose surface antigens had not been altered by germicidal chemicals. For example, we found that organisms exposed to 5% formalin for 30 min no longer reacted with their homologous antisera (H. G. Stoenner, unpublished observations). This finding suggests that protein must be a part of the antigens that characterize each serotype. Indeed, the differences among serotypes that we identified by serologic techniques have been confirmed in companion studies in which biochemical differences in antigenic structure have been elucidated by other techniques (20).

On the basis of current knowledge, it is impossible to predict how often *B. hermsii* can change its surface antigens. During experiments conducted to discover new types and to define serotypes emerging from each of the 24 types, 43 mice were eventually infected with new types. Had these, in turn, been similarly studied, additional new serotypes would likely have emerged from them. One is impressed with the large number of serotypes that may emerge from a given type (Table III), but it should be noted that some of those identified on day 7 may have emerged from variant types that arose early in the infection. It should also be noted that the variation in the number of emerging types was, in part, affected by the amount of growth in indicator mice; more serotypes were usually recognized in mice with a heavy spirochetemia.

Our findings on spontaneous conversion in cultures also indicate that a large number of serotypes can emerge from a single type. It is likely that all 11 serotypes seen in smears of eight cultures of type 21 arose from this type, but the possibility that some arose from type 24 or other more common variants cannot be firmly excluded. The ability of one type to change to many other types, and the fact that types 7 and 21 both commonly changed to 24, suggest a very complex genetic control of the mechanism. Our findings on spontaneous conversion late in this study explain our early difficulties in maintaining reasonably pure populations of some serotypes during

three to four passages in mice. For example, passages were made of four clones of type 16 before satisfactory antigen for immunizing mice was obtained. Unfortunately, in neither study on conversion were we able to calculate rates because we were unable to accurately count the number of variants in a given population. Also, the number of variants that grew in amplifier mice was affected by some cross-neutralization by the antiserum used to destroy the dominant population. Based on the prevalence of the variants, it was estimated that the rate was $\sim10^{-4}$–10^{-3} per cell per generation.

Our data do not suggest that *B. hermsii* converted in any definite order. In fact, it is clear that type 7 is dominant and that variants commonly revert to 7 in the absence of antibody to this serotype. This observation, however, may not reflect what happens in nature, because the organism in the tick from which the culture was started was type 1. Also, type 7 was not found in a spirochetemic patient from Colorado. (Heparinized blood had been submitted by Dr. I. M. Baird, Columbus, OH, to Dr. Willy Burgdorfer, RML, for diagnosis.) This patient, presumably bitten by *Ornithodoros hermsi*, had a mixed infection of 20, 24, and a new serotype (H. G. Stoenner, unpublished observations). It would be interesting to study the distribution of serotypes in more human cases and in naturally infected ticks.

The role of antibody in antigenic variation appears to be one of selection of variants and destruction of the dominant serotype population. The rate of antibody development in mice depends on the number of borreliae inoculated. We were able to detect serotype-specific immunoglobulin at 60 h PI, but not at 48 h, in mice inoculated with $\sim10^6$ borreliae (H. G. Stoenner, unpublished observations). However, on several occasions when we attempted to pass large inocula of serotypes at 48 h intervals, we noted that, in a single passage, a nearly pure population of one serotype changed to a mixture of variants that commonly emerge from that serotype. In these instances, we thought that sufficient antibody had developed and bound to the dominant population to destroy it in the next passage. In this regard, it should be noted that mice infected with *B. hermsii* have a marked lymphocytosis and consequently primed lymphocytes in a whole blood inoculum might alter the population during repeated passage.

Although we found that conversion occurs spontaneously and the process is not likely initiated by contact of the organism with early antibody, it appears that other host factors may be involved. For example, type 4 never emerged from types 7 and 3 in mice, but did so in rats (H. G. Stoenner, unpublished observations). When type 7 was inoculated into rats, nearly every rat, on the day after initial clearance, was also infected with a new serotype not seen in mice. In experiments on spontaneous conversion, there was general agreement between variant types seen in cultures and those that appeared in mice (Tables V and VI), but there were exceptions. Also, some serotypes that appeared in mice inoculated with 1,000 organisms did not appear in mice that received a 10-fold greater dose (Tables VI and VIII, tube 3). In these cases, perhaps cross-neutralization (for example, Table IV, J1-M3, 6th d inoculated with type 3) and varying rates of growth among serotypes determine which develop in the amplifier mouse.

Summary

At least 24 different serotypes were detected in populations of *Borrelia hermsii* that originated from a single organism. These serotypes were identified by staining with

specific fluoresceinated antisera prepared against cloned populations of living organisms of each type. In the order of decreasing frequency, the 10 types more often encountered were 7, which was clearly dominant, and 2, 17, 24, 13, 2, 1, 21, 11, and 12. Each of the 24 types were shown to change to 7 or more other serotypes.

Spirochetemia in mice was persistent, and relapses occurred when the concentration of organisms was sufficient for detection by visual means. After mice were inoculated with a single organism, peak spirochetemia usually occurred on day 4, after which clearance of organisms occurred, and an apparently pure population was replaced by a mixed population consisting of as many as seven variants. These types persisted for 2–3 d before being replaced by other types. Conversions occurred constantly and were independent of relapses. The rate of conversion in mice treated with cyclophosphamide to delay antibody production was comparable to that of controls. Spontaneous conversion was clearly demonstrated in tubes of fortified Kelly's medium inoculated with a single organism of type 7 or 21. 11 different variants appeared in eight cultures of type 21 by the time growth had reached 4×10^6–10^7 organisms/ml. The rate of spontaneous change was estimated to be $\sim 10^{-4}$–10^{-3} per cell per generation.

References

1. Obermeier, O. 1973. Vorkommen feinster eine Eigenbewegung zeigender Faden im Blute von Rekurrenskranken. *Zentralbl. Med. Wissenschaft.* 11:145.
2. Cunningham, J. 1925. Serological observations on relapsing fever in Madras. *Trans. R. Soc. Trop. Med. Hyg.* 19:11.
3. Schuhardt, V. T. 1942. The serology of the relapse phenomenon in relapsing fever. *In* Relapsing Fever in the Americas. F. R. Moulton, editor. American Association for the Advancement of Science, Washington, D. C. 58–66.
4. Felsenfeld, O. 1979. Borreliosis. *In* Handbook Series in Zoonoses. CRC Press, Inc., West Palm Beach. 1:79–96.
5. Kelly, R. T. 1971. Cultivation of *Borrelia hermsi. Science (Wash. D. C.).* 173:443.
6. Kelly, R. T. 1976. Cultivation and physiology of relapsing fever borreliae. *In* The Biology of Parasitic Spirochetes. R. C. Johnson, editor. Academic Press, Inc., New York. 87–94.
7. Coffey, E. M., and W. C. Eveland. 1967. Experimental relapsing fever initiated by *Borrelia hermsi.* I. Identification of major serotypes by immunofluorescence. *J. Infect. Dis.* 117:23.
8. Coffey, E. M., and W. C. Eveland. 1967. Experimental relapsing fever initiated by *Borrelia hermsi.* II. Sequential appearance of major serotypes in the rat. *J. Infect. Dis.* 117:29.
9. Gordon, M. A., M. R. Edwards, and V. N. Tompkins. 1962. Refinement of fluorescent antibody by gel filtration. *Proc. Soc. Exp. Biol. Med.* 109:96.
10. Thompson, R. S., W. Burgdorfer, R. Russell, and B. J. Francis. 1969. Outbreak of tick-borne relapsing fever in Spokane County, Washington. *JAMA (J. Am. Med. Assoc.).* 210:1045.
11. Stoenner, H. G. 1974. Biology of *Borrelia hermsi* in Kelly's medium. *Appl. Microbiol.* 28:540.
12. Peacock, M., W. Burgdorfer, and R. A. Ormsbee. 1971. Rapid fluorescent-antibody conjugation procedure. *Infect. Immun.* 3:355.
13. Green, J. H., S. B. Gray, Jr., and W. K. Harrell. 1976. Stability of fluorescent antibody conjugates stored under various conditions. *J. Clin. Microbiol.* 3:1.
14. Tully, J. G., R. F. Whitcomb, D. L. Rose, and J. M. Bove. 1982. *Spiroplasma mirum,* a new species from the rabbit tick (*Haemaphysalis leporispalustris*). *Int. J. Syst. Bacteriol.* 32:92.
15. Meynell, G. G., and E. Meynell. 1970. Theory and Practice in Experimental Bacteriology.

2nd Edition. Cambridge University Press, London 173–255.

16. Buchanan, R. E., and E. I. Fulmer. 1928. Physiology and Biochemistry of Bacteria. Williams and Wilkins Co., Baltimore. 19.

17. Santos, G. W. 1967. Immunosuppressive drugs (I). *Fed. Proc.* **26:**907.

18. Pickens, E. G., E. J. Bell, D. B. Lackman, and W. Burgdorfer. 1965. Use of mouse serum in identification and serologic classification of *Rickettsia akari* and *Rickettsia australis*. *J. Immunol.* **94:**883.

19. Philip, R. N., E. A. Casper, W. Burgdorfer, R. K. Gerloff, L. E. Hughes, and E. J. Bell. 1978. Serologic typing of rickettsiae of the spotted fever group by microimmunofluorescence. *J. Immunol.* **121:**1961.

20. Barbour, A. G., S. L. Tessier, and H. C. Stoenner. Variable major proteins of *Borrelia hermsii. J. Exp. Med.* **156:**1312.