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Autor:	Burgdorfer, W. / Aeschlimann, A. / Peter, O.
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¹ US Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana 59840, USA

² Zoological Institute of the University of Neuchâtel, Switzerland

***Ixodes ricinus*: vector of a hitherto undescribed spotted fever group agent in Switzerland**

W. BURGDORFER¹, A. AESCHLIMANN², O. PETER², S. F. HAYES¹, R. N. PHILIP¹

Summary

A tick/rickettsial survey in various parts of Switzerland revealed the presence of a new, hitherto undescribed spotted fever group rickettsia ("Swiss agent") in up to 11.7% of *I. ricinus* collected off vegetation. Infection in ticks was found to be generalized with rickettsiae developing intracellularly and occasionally also intranuclearly. As a result of massive growth in ovarian tissues, including the germinative cells, the rate of transovarial and filial infection was 100%.

The "Swiss agent" appears to be nonpathogenic for guinea pigs, domestic rabbits, and Swiss mice, but in male meadow voles (*Microtus pennsylvanicus*) it produces a microscopically detectable infection in the tunica vaginalis. The rickettsia grows well in tissue culture systems including chick embryo fibroblast, Vero, and vole tissue cells, when inoculated via yolk sac into 5-day-old hens' eggs, it kills 100% of the embryos after 5 to 7 days.

Antigenic relatedness of the "Swiss agent" to rickettsiae of the spotted fever group was indicated by indirect and direct fluorescent antibody staining. Preliminary serologic typing by microimmunofluorescence and by microagglutination indicated that the "Swiss agent" differs from all prototype strains of spotted fever group rickettsiae studied so far.

Key words: tick; *Ixodes ricinus*; rickettsia; spotted fever group.

Correspondence: Prof. Dr. A. Aeschlimann, Zoological Institute of the University of Neuchâtel, CH-2000 Neuchâtel 7

Introduction

The only rickettsial disease of man recognized in Switzerland is Q fever. Every year about 125 serologically confirmed cases come to the attention of the public health authorities. Although it is generally assumed that ticks are important means by which *Coxiella burnetii*, the causative agent of Q fever, is transferred from natural foci to domestic animals, there is no evidence that ticks in Switzerland are responsible for transmitting this rickettsia to man. All cases, to the best of our knowledge, are being contracted as the result of close association with livestock, particularly sheep, goats, and cattle. Recent discoveries of *C. burnetii* and of spotted fever group rickettsiae in ticks from southern Germany (Liebisch, 1977; Řeháček et al., 1977; Liebisch et al., 1978) and from Austria (Kaaserer et al., 1976; Bazlikova et al., 1977) prompted the Zoological Institute of the University of Neuchâtel to initiate a long-term project to determine the role of ticks as vectors of rickettsiae and other microorganisms in Switzerland (Aeschlimann et al., 1979)³. This paper presents preliminary data on the occurrence of a hitherto undescribed spotted fever group rickettsia, hereafter referred to as "Swiss agent", in up to 11.7% of *Ixodes ricinus* collected from various parts of Switzerland.

Material and methods

From May through July, 1978, several known foci of *I. ricinus* in the cantons of Neuchâtel, Bern, Zug, Aargau, and Zürich were visited, and ticks were collected by flagging. The ticks were examined for rickettsiae as follows: nymphs were crushed individually on microscope slides and their tissues were stained by Giménez' (1964); adults were subjected to the hemolymph test (Burgdorfer, 1970). All hemolymph test-positive males and some positive females were dissected for the preparation of multiple tissue smears. Some were stained by Giménez', others were treated with fluorescein isothiocyanate-labelled immune sera to *Rickettsia rickettsii* (Wachsmuth-74), *R. conorii* (Simko); *R. prowazekii* (ZRS), and *C. burnetii* (Ohio) to obtain preliminary identification of the rickettsiae.

Many hemolymph test-positive female ticks were fed together with normal males on domestic rabbits and/or guinea pigs. Upon repletion, the females were stored separately in vials and were allowed to lay eggs. We speculated that the rickettsiae present in these ticks would be passed transovarially to the progeny. From each line, the F_1 larval ticks were eventually fed on Swiss mice, and the resulting nymphs were used for isolation and characterization of the agent.

For isolation of the rickettsiae, tissues of spent females or of freshly molted F_1 nymphs were triturated in 2.0 ml of cold brain heart infusion broth (BHI), and 0.25 ml of the suspension was injected intraperitoneally into each of four male meadow voles (*Microtus pennsylvanicus*). The remaining inoculum, frozen at -65°C , was stored as reference material. Beginning at day 3 after inoculation, one or two voles per day were killed and multiple smears were prepared from scrapings of their tunica vaginalis. These were stained by Giménez' method and were examined for rickettsiae.

Limited studies were also carried out to determine the behavior of the "Swiss agent" in chick embryos and in cell cultures. For this purpose, tunica vaginalis and spleens of infected voles were

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triturated in BHI and aliquots of 0.25 to 0.5 ml were injected into the yolk sacs of 5-day-old embryos or into monolayers of chick embryo fibroblasts, Vero cells, and cell lines derived from embryonic or tunica vaginalis tissues of voles (Burgdorfer and Mavros – unpublished data). The methods used for maintaining and inoculation cell cultures were similar to those reported by Cory et al. (1974).

Indirect microimmunofluorescence (MIF) and microagglutination (MA) tests were used to determine serologic responses of Swiss mice, infected with the “Swiss agent” either by inoculation or by feeding of infected ticks, to various rickettsial antigens. In the MIF test (Philip et al., 1978), these antigens included: the “Swiss agent” ($C_9 P\varphi_9$), *R. rickettsii* (“R”; “Hlp”), *R. conorii* (BF), *R. sibirica* (No. 246), *R. slovaca* (“B” and “D”), *R. parkeri* (Mississippi), *R. montana* (M_{5-6}), *R. australis* (Phillips), *R. akari* (No. 29), *R. rhipicephali* (3–7–♀6), *R. prowazekii* (Breinl), *R. typhi* (Wilmington), and *R. canada* (No. 2678).

In the MA test, antigens prepared according to Ormsbee et al. (1978), included: the “Swiss agent” ($C_9 P\varphi_9$), *R. rickettsii* (Camas ♀165; Hansen-73; Sawtooth ♀2), *R. montana* (M_{5-6}), *R. conorii* (Simko), *R. rhipicephali* (3–7–♀6), *R. sibirica* (No. 246), *R. prowazekii* (ZRS), *R. typhi* (Wilmington), and *R. canada* (No. 2678). The test was performed according to Fiset et al. (1969), with the exception that an isotonic saline buffer (pH 7.0) with Tris (hydroxymethyl) aminomethane was mixed with bovine serum albumin and used as diluent to facilitate agglutination.

Lastly, the ultrastructure of the “Swiss agent” in tick tissues was determined by procedures described elsewhere (Hayes and Burgdorfer, 1979).

Results

As summarized in Table 1, a total of 4,092 nymphal and adult *I. ricinus* was collected from vegetation in 7 tick-infested areas. Of these, 344 (8.4%) were positive for a rickettsialike organism. The prevalence of infected ticks varied from 1.8 to 11.7% depending on the site of collection. In Giménez' stained hemolymph or tissue smears, the organisms appeared faintly pink and predominantly diplococcal or rodshaped (Fig. 1). Occasionally, a tick was recorded as being infected with long, bacilluslike or threadlike organisms. All positive ticks showed moderate to massive generalized intracellular infections of their tissues. Intranuclear growth was regularly seen in the tissues of the rectal ampule (Fig. 2) and in those of the male genital organs. Rickettsial infections were also heavy in ovarian tissues including the oogonia and oocytes. Each of 50 randomly selected eggs and larvae from each of 25 positive females proved to be infected, as shown by transmission to the progeny.

None of the Swiss mice, guinea pigs, or domestic rabbits responded to injected suspensions or to feeding of infected ticks with elevated temperatures, splenomegaly, or microscopically detectable infections in tissues. Male meadow voles, on the other hand, developed pronounced splenomegaly and invariably had rickettsial growth in their tunica vaginalis as early as 3 days after inoculation (Fig. 3). However, serial passages of the “Swiss agent” in meadow voles have been unsuccessful.

So far isolates from five different tick lines have been established in chick embryos. In each case, numbers of rickettsiae in yolk sac tissues were moderate but killed 100% of the embryos in 5 to 7 days after inoculation.

The “Swiss agent” grew well and produced massive infections in all cell

Table 1. Rickettsialike organisms in *Ixodes ricinus* from vegetation in various parts of Switzerland, 1978

Site	Number of ticks		Percentage
	examined	infected	
Bois d'Hôpital (Neuchâtel) . . .	100 NN*	3	3.9
	310 AD**	13	
Staatswald (Bern)	520 NN	38	8.3
	1,324 AD	115	
Le Chablais (Fribourg)	650 AD	60	9.2
Seewald (Bern)	273 NN	27	11.7
	527 AD	68	
Schachenwald (Zug)	34 NN	2	6.7
	219 AD	15	
Sins (Aargau)	122 AD	2	1.8
Kappel (Zürich)	13 AD	1	7.7
Totals	4,092	344	8.4

* NN = nymphal ticks, Giménez-stained tissues

** AD = adult ticks, hemolymph test

culture systems used (Fig. 4); however, there was little cytopathogenicity and no evidence of plaque formation.

The ultrastructure of the "Swiss agent" is similar to that of *R. rickettsii* and other spotted fever group agents (Fig. 5). The cytoplasmic matrix has a pale, at times splotchy almost vacuolar appearance; it is fibrillar and reticular and contains rather nondistinct ribosomes. Where present, the slime layer is much less prominent than in other pathogenic rickettsiae. Organisms often are closely associated with the cytoplasmic components of the host cell. The cell wall shows some minor variations from that of other rickettsiae of the spotted fever and typhus groups. The outer leaflet is thicker (2–4 nm) and the inner layer is not as osmophilic as it is in other rickettsiae. The periplasmic space appears to be uniform in size, and the plasma membrane, in general, is not as sinuous as seen in other rickettsiae.

Limited testing by MA and MIF of sera from Swiss mice inoculated with infected tick suspensions or fed upon by infected F₁ larvae revealed highly specific homologous reactions with little or no cross reaction against any other rickettsial antigen (Table 2). Nevertheless, antigenic relationship of the "Swiss agent" to the spotted fever group was indicated by direct fluorescent antibody staining. Invariably, conjugates against *R. rickettsii* or *R. conorii*, gave a particulate, dustlike staining pattern suggesting reaction(s) with certain antigenic components of the agent. Similarly, homologous conjugates prepared from sera of

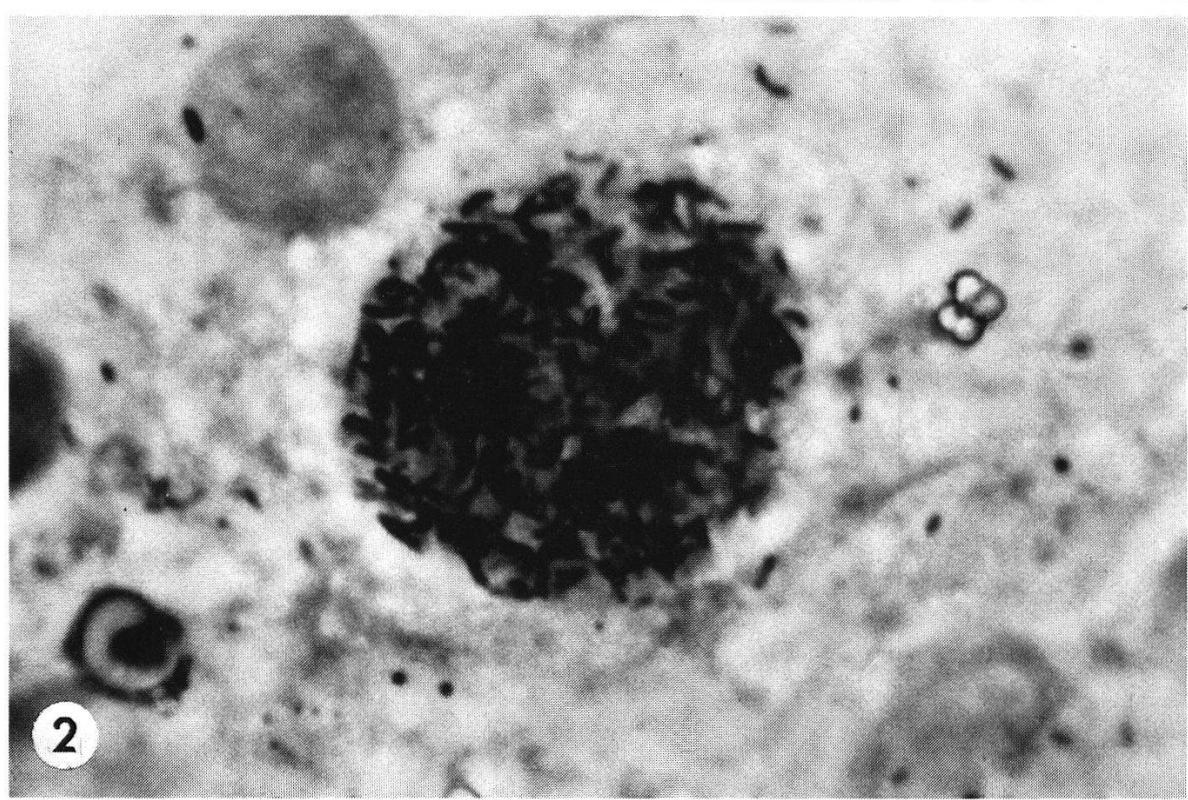
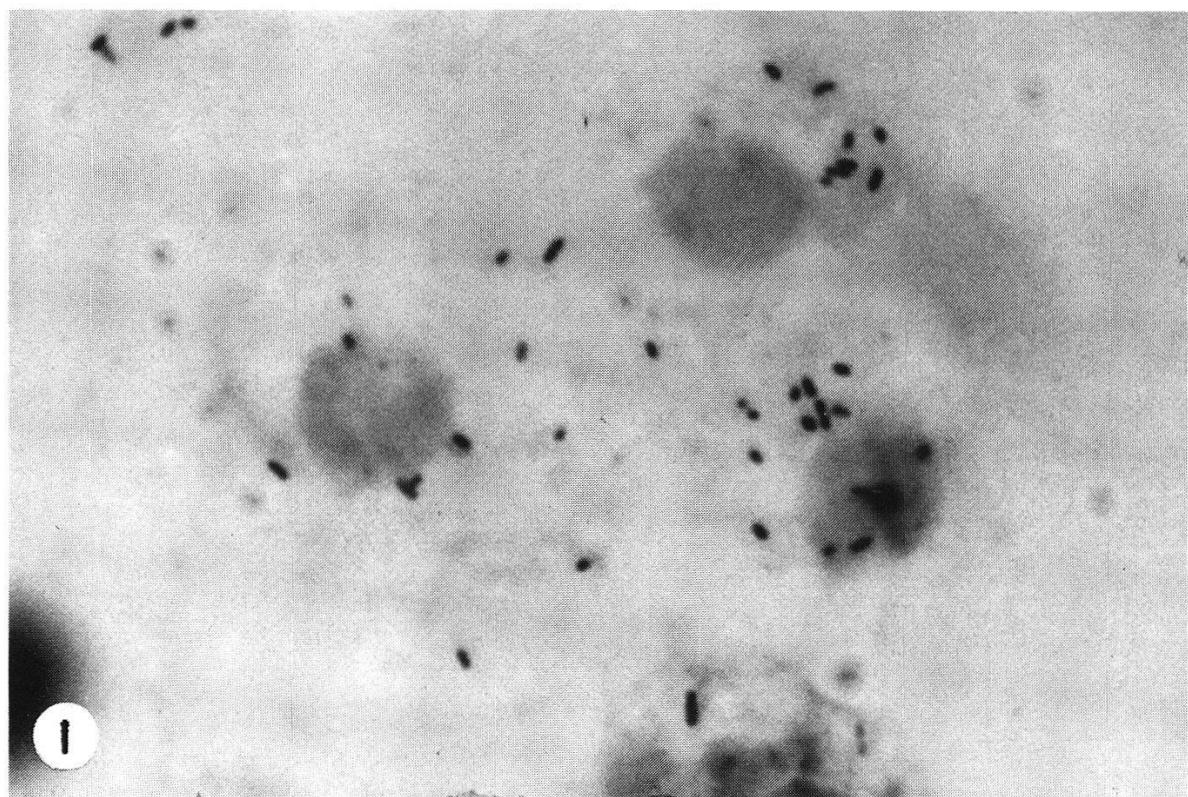


Fig. 1. Appearance of the "Swiss agent" in hemocytes of infected adult *Ixodes ricinus* from Staatswald (Bern) (Giménez stain, 1,900 \times).

Fig. 2. Intranuclear growth of the "Swiss agent" in rectal ampule tissue of infected *I. ricinus* (Giménez stain, 1,900 \times).

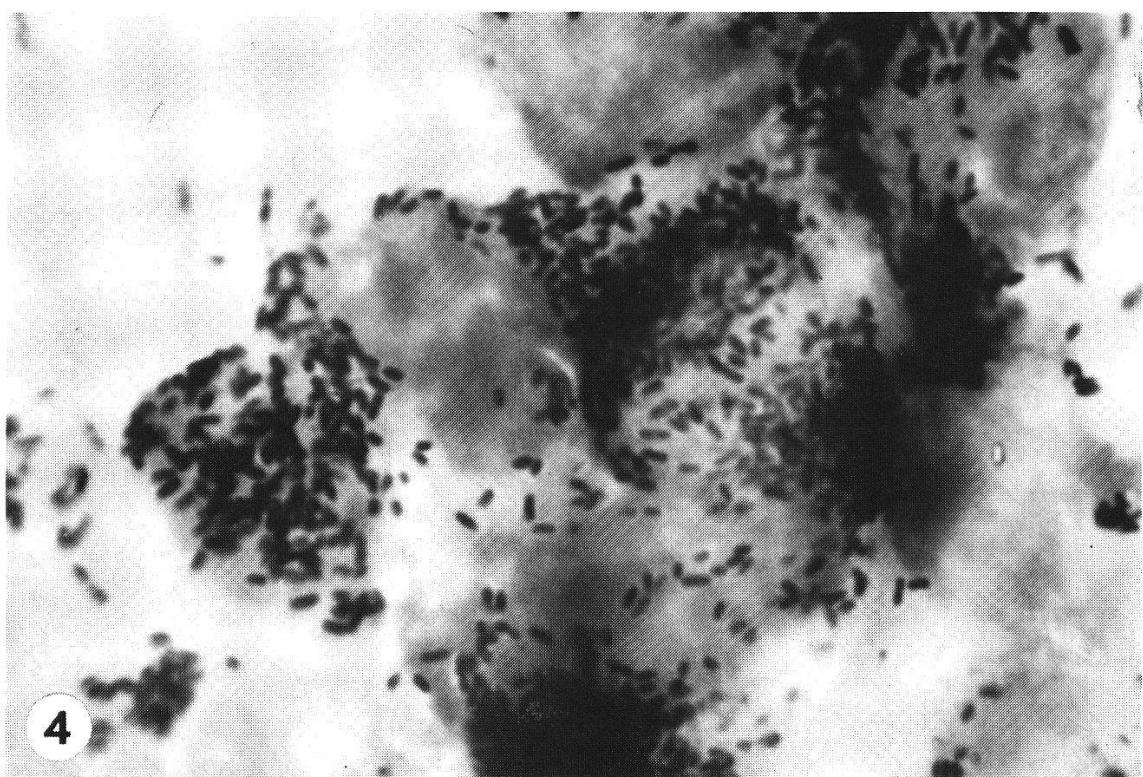
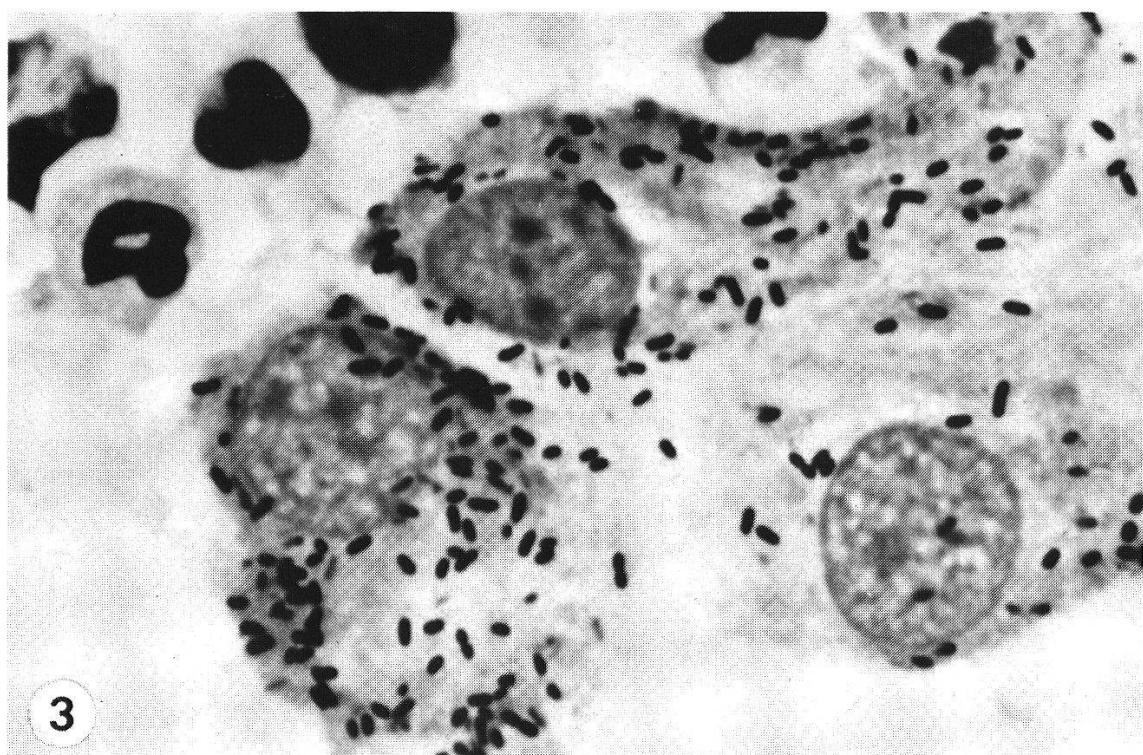


Fig. 3. "Swiss agent" rickettsia in tunica vaginalis of *Microtus pennsylvanicus* (Giménez stain, 1,900 \times).

Fig. 4. Massive growth of the "Swiss agent" in a cell line of tunica vaginalis from *M. pennsylvanicus* (Giménez stain, 1,900 \times).

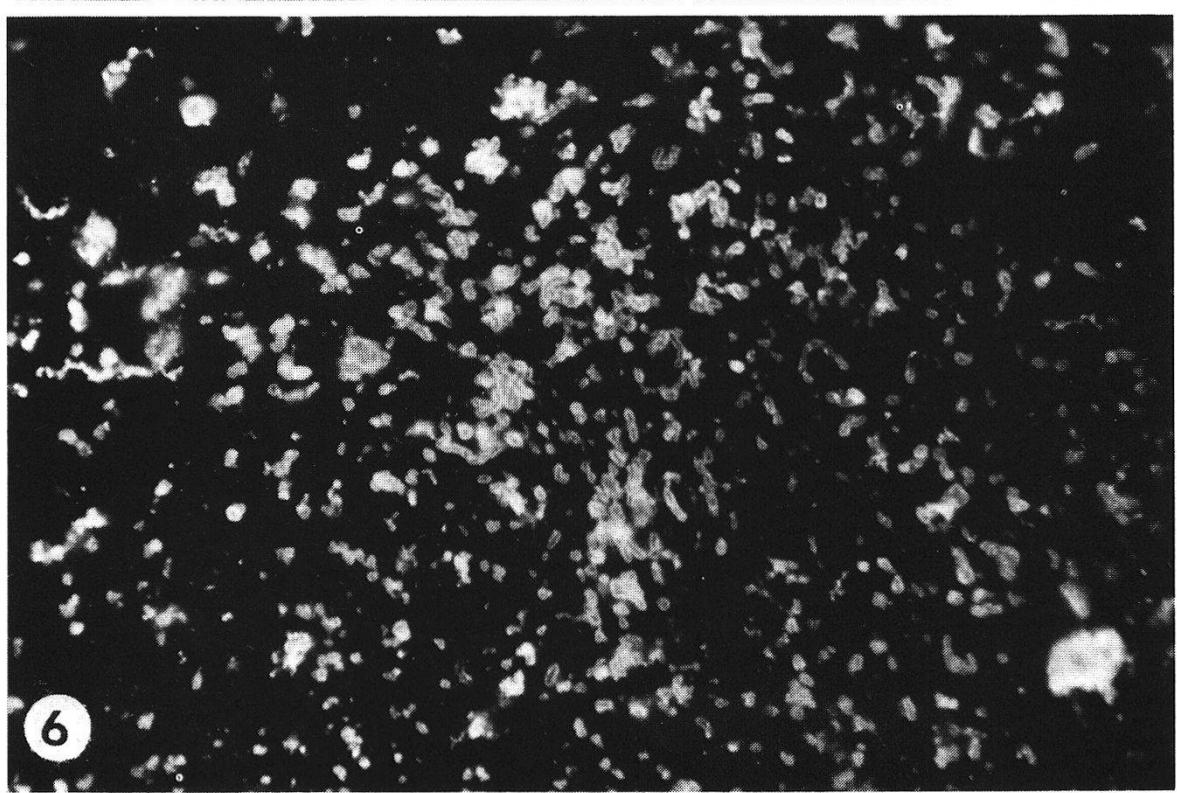
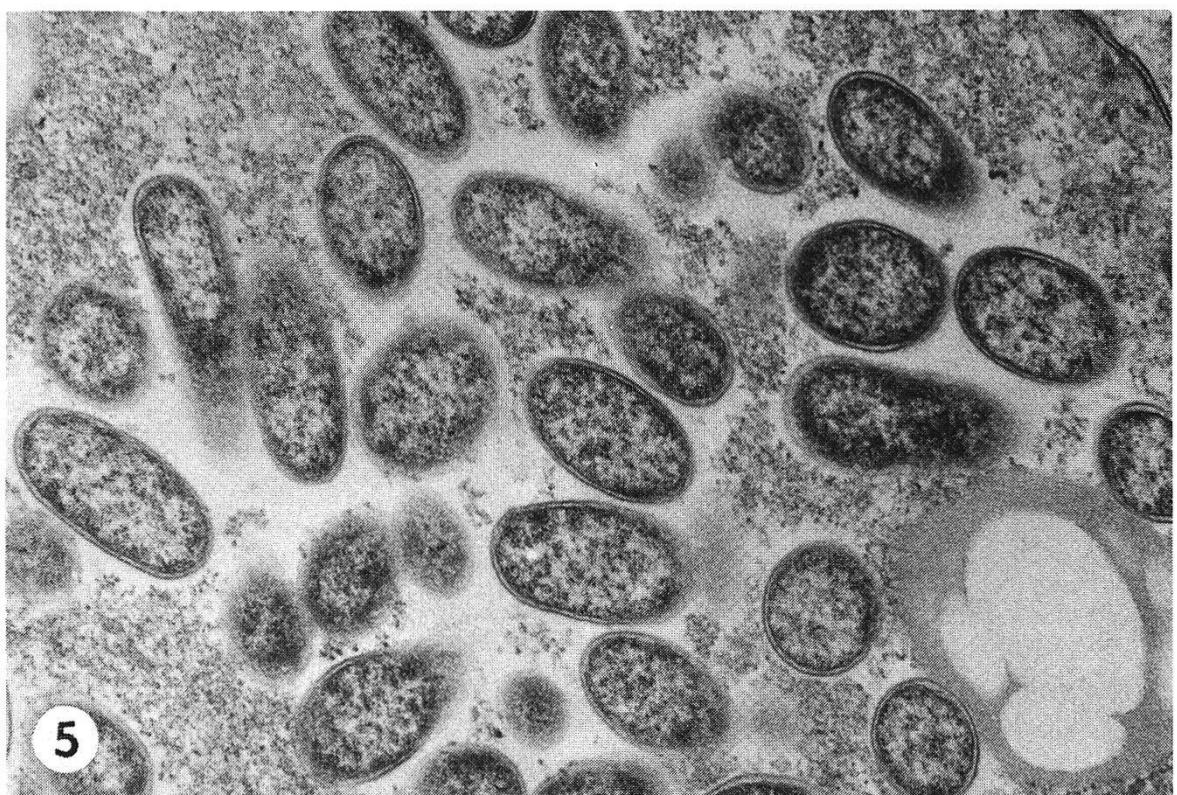


Fig. 5. Electromicrograph of the "Swiss agent" in Malpighian tubule tissue of engorged nymphal *I. ricinus* (26,300×).

Fig. 6. Indirect fluorescent antibody reaction of the "Swiss agent" with convalescent serum from a person affected with Rocky Mountain spotted fever (1,100×).

Table 2. Identification of the "Swiss agent" in MIF-cross tests of mouse sera*

Antisera	Antigens				
	Swiss agent (110)**	<i>R. rickettsii</i> (35)	<i>R. conorii</i> (51)	<i>R. slovaca</i> (81)	<i>R. sibirica</i> (3)
"Swiss agent" (110) . . .	128***	0	0	0	0
<i>R. rickettsii</i> (35)	0	128	0	32	16
<i>R. conorii</i> (51)	0	tr****	128	32	32
<i>R. slovaca</i> (81)	0	16	32	256	128
<i>R. sibirica</i> (3)	0	64	64	512	512

* Results with additional antigens cited in the text were similar to those tabulated here.

** Figures in parentheses denote code numbers of rickettsial strains.

*** serum dilution endpoint

**** trace of reaction at 1:8 dilution

Table 3. Spotted fever group relationship of "Swiss agent" by direct FA staining

Antigens	"Swiss agent" conjugate							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
"Swiss agent" (110)	+	+	+	+	+	+	+	(+)
<i>R. rickettsii</i> (35)	+	+	+	+	(+)			
<i>R. montana</i> (9)	+	+	+	(+)				
<i>R. sibirica</i> (3)	+	+	+	(+)				
<i>R. slovaca</i> (81)	+	+	+	(+)				
<i>R. akari</i> (8)	+	+	(+)					
<i>R. conorii</i> (51)	+	+	(+)					
<i>R. rhipicephali</i> (12)	+	(+)						

+= good fluorescence

(+)= weak fluorescence

meadow voles immunized by inoculation with infected tick suspensions reacted with several prototypes of spotted fever group rickettsiae (Table 3). Specific reactions against the "Swiss agent" were obtained only after 32-fold dilution of the preparation. Antigenic relatedness to the spotted fever group rickettsiae was also shown in the indirect FA test. Human immune sera to *R. rickettsii* reacted strongly with the "Swiss agent", as illustrated in Fig. 6.

Discussion

The presence of spotted fever group rickettsiae in *I. ricinus* has been reported from several European countries. Giroud et al. (1962) noted rickettsialike

microorganisms in four pools of this tick taken off domestic animals in the eastern part of France. They identified them serologically as *R. conorii*. In a subsequent survey in various parts of that country, 33 of 71 pools of *I. ricinus* contained morphologically typical or atypical rickettsiae that could not readily be adapted to chick embryos or other laboratory animals. However, sera of animals inoculated with these rickettsiae, had antibodies against *R. conorii*, *C. burnetii*, and the psittacosis group agents (Giroud et al., 1965). Additional evidence of spotted fever group rickettsiae (*R. conorii*) in *I. hexagonus*, *Dermacentor reticulatus*, and *D. marginatus* from southeastern parts of France was reported by Gilot (1975).

During extensive investigations on tick-borne rickettsioses in various parts of Slovakia from 1968–1974, and more recently in south Bohemia, Řeháček and associates (1972; 1975; 1976a; 1976b; 1977) examined 4,441 adult *I. ricinus* by the hemolymph test and found 171 infected with rickettsiae. Most were identified serologically or by direct immunofluorescence as members of the spotted fever group. No characterization of these organisms was reported. In the 1968–1970 study, Řeháček et al., (1972) obtained 3 rickettsial isolates in chick embryos. They multiplied poorly but killed the embryos in 3 to 4 days after inoculation. Guinea pigs were said to respond only exceptionally with short and weak febrile reactions. There was moderate scrotal involvement, but this manifestation was seen also in guinea pigs without fever.

Recent tick/rickettsial surveys in Bulgaria revealed extremely high (up to 52.2%) percentages of *I. ricinus* infected with spotted fever group rickettsiae (Georgieva et al., 1976; Georgieva and Kyossev, 1978). These findings were based on positive hemolymph tests and on subsequent identification of the rickettsiae by direct immunofluorescence. Guinea pigs inoculated with suspensions of 5 infected adult *I. ricinus* responded in at least 4 instances with CF antibodies to spotted fever group antigens.

Thus, rickettsiae detected so far in *I. ricinus* and in many other species of European ticks (see references cited) have been identified in preliminary ways only by serologic tests and/or immunofluorescence. Their precise antigenic relation to the prototype strains of tick-borne rickettsiae remains to be established. An exception to this is a spotted fever group agent isolated from *D. marginatus* in Slovakia (Brezina et al., 1968). When subjected to serologic tests, this organism was found to differ so much from other prototype species of the spotted fever group that it was considered a new species, for which the name, *R. slovacca*, was proposed (Urvölgyi and Brezina, 1978).

The “Swiss agent” also appears to be a hitherto undescribed member of the spotted fever group. Its antigenic relationship to this group is indicated by cross reactions in direct and indirect immunofluorescent staining. Also typical for spotted fever group rickettsiae is its growth pattern in the tick vector in which it produces generalized infection, its ability to invade and multiply in nuclei, and its transmission via eggs to the progeny.

Serologically, at least in MA and MIF tests, the "Swiss agent" is quite distinct in that it does not cross-react with any of the prototypes of spotted fever group rickettsiae. In spite of these rather unique biological characteristics, we do not now propose a scientific name for the "Swiss agent". This must await results of experiments in progress to determine the guanine plus cytosine composition of the rickettsia's DNA, its protein patterns by SDS-polyacrylamide gel electrophoresis, its antibiotic sensitivity, and the immune responses of laboratory animals as measured by other serologic tests. Also underway are investigations pertaining to the ecology of this tick-borne agent as well as its potential significance as a pathogen for man.

Lastly, it should be noted that none of the 3,165 adult *I. ricinus* examined was infected with *C. burnetii*, although all these ticks originated from areas where human cases of Q fever occurred in association with the livestock industry. Detection of natural foci of this agent would not be surprising in view of reports from Slovakia (Řeháček et al., 1970; 1975), Austria (Kaaserer et al., 1976), and Bulgaria (Georgieva et al., 1976; Georgieva and Kyossev, 1978) where *I. ricinus* and other species of ixodid ticks were found to be infected with *C. burnetii*.

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