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ANTIGENIC RELATIONSHIPS AMONG SEVERAL LIMAX AMOEBAE ISOLATES ASSESSED WITH THE INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

L. ČERVA and J. KRAMÁŘ

Army Institute of Hygiene, Epidemiology and Microbiology, Prague; Department of Parasitology, Faculty of Natural Sciences, Charles University, Prague

Abstract. Indirect immunofluorescence was used for the detection of relationships among four strains of pathogenic amoebae of the genus *Naegleria* (HB-1, Vitek, 0359, 0360), and three species of the genus *Acanthamoeba* (*A. castellanii*, *A. culbertsoni*, *A. polyphaga*). We found strong cross reactions in all strains of *Naegleria* identified as the pathogenic agent of primary amoebic meningoencephalitis in man, this evidence suggesting their antigenic uniformity. By contrast, antigenic differences of considerable extent wer found in the three species of the genus *Acanthamoeba*; cross reactions were restricted to the lowest serum dilutions only. The sensitivity and specificity of the IFAT may facilitate the differentiation of *limax* amoebae.

The interest in the study of free-living *limax* amoebae has increased in view of recent reports indicating that some of these organisms are responsible for lethal meningoencephalitis in man, or are pathogenic for laboratory animals.

Goldman et al. (1960b) used fluorescent antibody in their antigenic analysis of *Entamoeba histolytica* and *E. hartmanni*. Goldman (1960a) constructed a microfluorimeter for the objective measurement of fluorescence of amoebae with FITC conjugated gamma globulin against homologous and heterologous strains. Goldman and Gleason (1962) employed a technique based upon absorption of conjugates of heterologous strains in order to assess antigenic relationships of two strains of *Entamoeba histolytica* and one strain of *E. hartmanni*. Also Siddiqui and Balamuth (1965) used fluorescent antibody for the differentiation of several parasitic and free-living strains of amoebae, but evaluated their results by unaided vision only and confirmed them with Ouchterlony's precipitation reaction or with the reaction for an acetocellulose membrane. Červa (1966) employed successfully both direct and indirect fluorescent antibody tests for the detection of pathogenic amoebae (*Acanthamoeba culbertsoni*) in the tissues of experimentally infected mice. Honigberg and Goldman (1968) used quantitative fluorescent antibody methods for the immunological analysis of the effect of prolonged culture of *Trichomonas gallinae*. Augustine and Lund (1970) compared two strains of *Histomonas meleagridis* and one strain of *H. wenrichi* by means of the indirect fluorescent antibody test; they evaluated their results by unaided vision only.

Having no microfluorimetric equipment, we obtained a good reproducibility of the values by means of a simplified scale employed for the estimation of fluorescence intensity. This enabled the determination of serum antibody titers during reactions with homologous and heterologous strains.

MATERIAL AND METHODS

Antigens: we studied 7 strains of amoebae cultured for several months or years under axenic conditions. *Acanthamoeba castellanii*—isolated originally by Castellani (1930) and axenized in our laboratory in 1964. *Acanthamoeba culbertsoni* (syn. *A. castellanii* strain Al), showing marked antigenic properties for laboratory animals. Isolated by Culbertson et al. (1958) from a culture of monkey kidney cells and described as the first pathogenic strain of *limax* amoebae. *Acanthamoeba polyphaga*, strain 0ll—isolated from the water of a swimming pool (Červa 1971); it is feebly pathogenic for laboratory mice upon intracerebral and intranasal inoculation. *Naegleria* sp., strain 11B-1, the first strain isolated from the liquor of man infected with primary amoebic meningoencephalitis (PAME) in the U.S.A. (Butt et al. 1968, Culbertson et al. 1968). *Naegleria* sp., strain Vítek, isolated from a case of primary amoebic meningoencephalitis in North Bohemia in 1968 (Červa et al. 1969). *Naegleria* sp., strain 0359 and 0360, isolated by Jadin et al. (1971) from two cases of PAME in Antwerp (Belgium).

All *Naegleria* strains are highly pathogenic for laboratory animals. The original strain, *Acanthamoeba castellanii*, was cultured at room temperature, the remaining strains at 35 and 37 °C, in the same medium containing 2 % Bacto Casitone Difco and 10 % fresh horse serum in distilled water (Červa 1969). Well-grown cultures aged 4—5 days (10—14 days with *A. castellanii*) were centrifuged for 10 min at 2,000 rpm/min; the supernatant was removed and drops of thick sediment were placed on microscopic slides and slightly flattened. When dry, the smears were fixed with acetone p.a. and left until all liquid had evaporated. The antigens fixed on the slides were kept at -20 °C to be ready for use.

Antisera: these were derived from rabbits immunized by repeated intravenous injection of washed culture centrifugate of the individual strains (4—5 doses at weekly intervals). We used viable *Acanthamoeba* and frost-killed *Naegleria*, using viable *Naegleria* for the last dose only. The number of amoebae per dose ranged from 2—5 . 10^7. Antisera were kept at —20 °C.

Conjugate for the IFAT: FITC labelled swine-gamma globulin against rabbit gamma globulin produced commercially by SEVAC, Prague, trade mark SwAR.

The slides with the antigen were taken out of the freezer and left to dry at room temperature. Rabbit sera (antisera) were diluted with saline (from 1 : 10 in a twofold series); 30 min incubation with the antigen at 37 °C in the moist chamber, rinsing of serum in tap water and of slides in saline solution (pH 7.2, 0.01 M phosphorus) for 15 min in the automatic shaker, another rinse in tap water for 5 min, then kept at room temperature until all water had evaporated. Conjugate applied for 30 min at 37 °C in the moist chamber (staining titer 1 : 10), Evans's blue, dilution 1 : 10,000 added. The slides were rinsed in the way described for rinsing after exposure to serum, and embedded in buffered glycerol.

Controls: control staining was performed with a standard positive and a standard negative serum; in addition, antigen was exposed to conjugate without a specific serum.

The results were read with the Soviet luminescent microscope ML-2 in incident light with a high pressure mercury tube DRŠ-250. Excitation filter FS-L-2 connected with filters BS-8-2 and STS-7-2, barrier filter no. 2 (ŽS-18 connected with the filter ŽZS-19). Immersion objective 90× eye pieces in the binocular head 4×. Guide-lines employed for the estimation of fluorescence, intensity:

0..... red stained amoebae with no sign of fluorescent rim
±..... the red staining interrupted by an occasional green fluorescent rim
+..... the red-stained amoebae surrounded by a thin, uninterrupted, green, fluorescent rim
++..... the same as under +, except that the rim is thicker
+++.... thick, green, fluorescent rim of intensive brightness; the green coloration of the remaining body surface is less intensive

Determination of antiserum titer: we considered the antiserum to be the last converted value of the dilution still giving a positive reaction to +.

RESULTS

As indicated by the results of our preliminary experiments antigens containing morphologically uniform individuals were found to be the most suitable ones for the evaluation of antiserum titres. The excessive polymorphism of amoebae in either too young or older cultures showing differently advanced stages of protozoan degeneration, makes it difficult to assess the limits of positivity. Also praecystic stages and cysts which

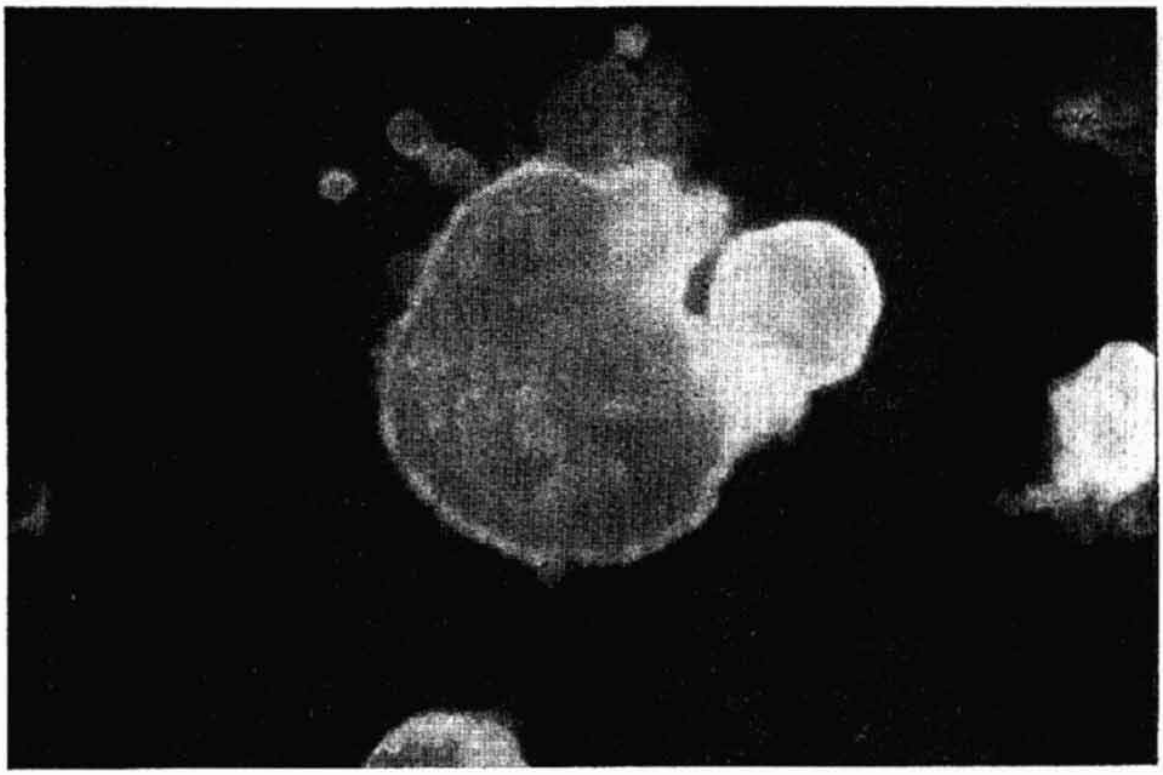

Fig. 1. *Naegleria* sp., strain Vítek—positive IFAT. Immersion objective 90×, eye pieces 4×

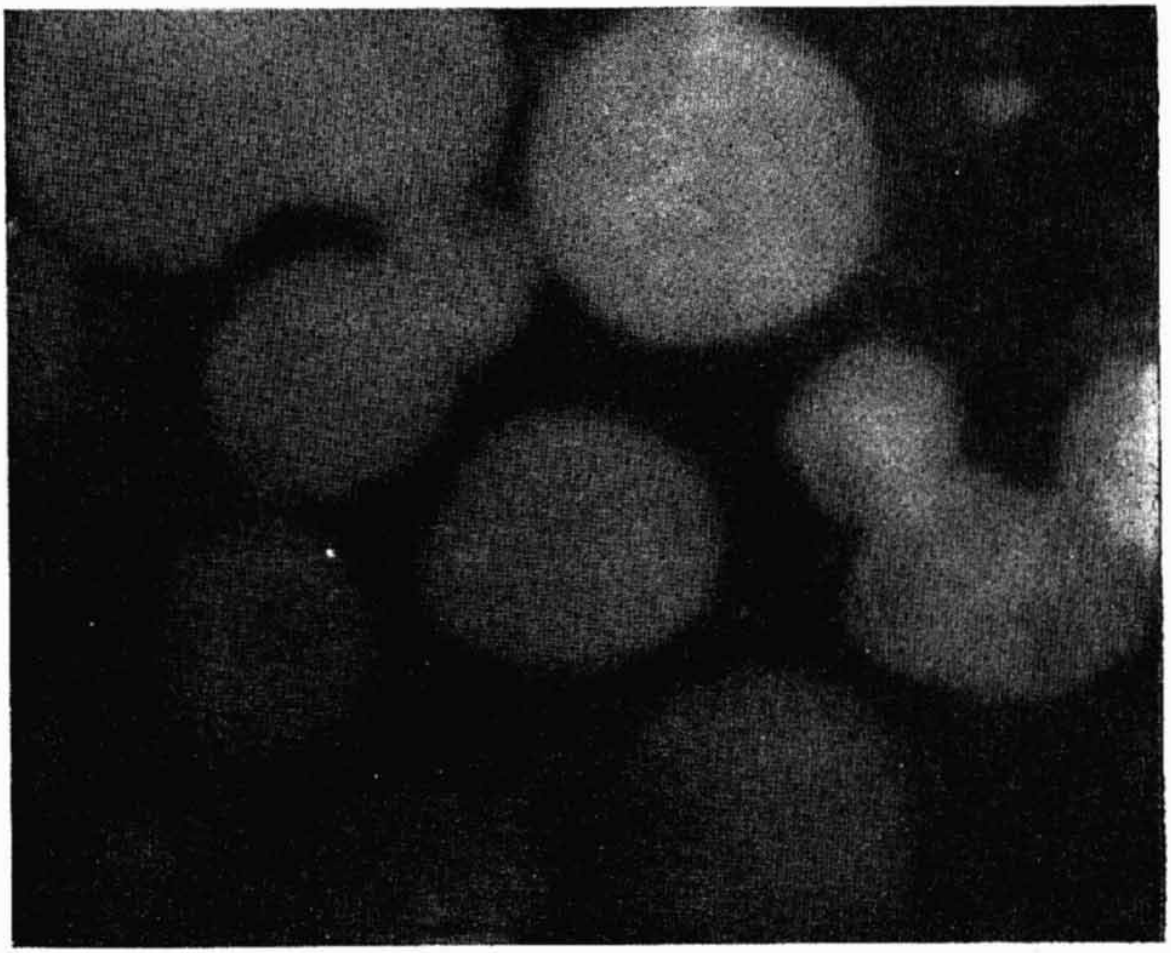

Fig. 2. *Naegleria* sp., strain Vítek—negative IFAT. Immersion objective 90×, eye pieces 4×

may be found in culture of several of the strains under consideration, constitute a source of unspecific positive reactions in spite of the fact that their fluorescence shows the typical shade of yellowish green colour. The results of antisera and antigen reactions are shown in Table 1. In the three *Acanthamoeba* strains, the IFAT disclosed distinct differences in the height of titers of positive reactions with homologous and heterologous sera. Cross reactions associated with generic relationships among the strains were restricted to the lowest serum dilutions only. No traces of common antigenic components were disclosed in sera against *Naegleria*, and their antigens did not stain with rabbit sera immunized with *Acanthamoeba* even at the lowest possible dilution.

The four *Naegleria* strains differed considerably from the tested *Acanthamoeba* strains in strong cross positivity of antigens and antisera; no differences, however, were found in the titers of reaction of heterologous and homologous components. This remarkable antigenic uniformity of pathogenic *Naegleria* from the American and European continents indicates their possible identity.

DISCUSSION

In this contribution intended to introduce a series of studies on the antigenic structure of *limax* amoebae by means of the IFAT, we are presenting a comparison of a small portion of amoebic strains from our culture collection. The strains chosen for this study grow well under axenic conditions in a simple uniform medium, enabling thus maximum conditions for a comparison of the results of serological tests and excluding the disturbing interference of different components of the culture media on the quality of the antigens. Moreover, these strains are suitable for mass production of a pure antigen satisfactory for the preparation of antisera and slides employed for the pertinent reactions.

The results of the comparison of three *Acanthamoeba* strains which are almost identical in their morphology and, hence, difficult to be distinguished from one another with standard panoptic staining methods indicate that the IFAT is a highly specific and sensitive method. These results, consistent with those of Adam's (1964) immobilisation tests and Červa's (1967) complement-fixation tests, are favouring the view that

Table 1. Results of cross reaction by the IFAT in *limax* amoebae

Antigen		Antisera						
Species	Strain	Castel.	A_1	011	HB_1	Vítek	359	360
Acanthamoeba castellanii	*Castellani*	640	0—10	10	0	0	0	0
Acanthamoeba culbertsoni	A_1	0—10	160	0—10	0	0	0	0
Acanthamoeba polyphaga	011	0—10	0—10	160	0	0	0	0
Naegleria sp.	HB_1	0	0	0	160	320	160	320
	Vítek	0	0	0	160—320	320	80—160	320
	359	0	0	0	320	320	160	320
	360	0	0	0	160	160	160	320

Acanthamoeba culbertsoni is an independent species. Until recently, this species was recorded as a pathogenic strain of *Acanthamoeba* (*Hartmannella*) *castellanii*. The disclosure of distinct differences in the antigenic structure of *Acanthamoeba* is evidently due to an improvement of the IFAT, in comparison with earlier results (Červa 1966) which were not obtained with a quantitative method.

On the other hand, the consistence of titers of the cross reactions among pathogenic *Naegleria* is amazing in view of the high sensitivity and specificity of the IFAT. It is contradictory to the suggestion to list the individual isolates causing PAME as separate species, e.g., *Naegleria fowleri*, *N. aerobia*, *N. invadens*, etc. Therefore, it should be attempted to asses with the IFAT the possible differences between pathogenic and nonpathogenic *Naegleria* strains.

АНТИГЕННЫЕ ВЗАИМООТНОШЕНИЯ МЕЖДУ НЕКОТОРЫМИ ВЫЯВЛЕННЫМИ ШТАММАМИ АМЕБ ТИПА »limax« С ПОМОЩЬЮ НЕПРЯМОГО МЕТОДА ФЛУОРЕСЦИРУЮЩИХ АНТИТЕЛ

Л. Черва и Й. Крамарж

Резюме. Непрямым методом флуоресцирующих антител изучались взаимоотношения между 4 штаммами патогенных амеб рода *Naegleria* (HB-1, Vítek, 0359 и 0360) и 3 видами рода *Acanthamoeba* (*A. castellanii*, *A. culbertsoni*, *A. polyphaga* 011). Антисыворотки изготовлялись путем иммунизации кроликов амебами из аксенических культур. На фиксированный ацетоном на предметных стеклах антиген воздействовали серией разведенных гомологичных и гетерологичных антисывороток. Все штаммы рода *Naegleria*, выявленные в качестве возбудителей первичного амебного менингоэнцефалита человека, давали значительные перекрестные реакции, свидетельствующие об единстве антигенов. Исследуемые три вида рода *Acanthamoeba* четко отличались друг от друга в отношении антигенов. Проводились лишь перекрестные реакции с самым низким разведением сыворотки. Чувствительность и специфичность непрямого метода иммунофлуоресцирующих антител могут облегчить видовую дифференцировку амеб группы „limax".

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L. Č., Vojenský ústavy hygieny, epidemiologie a mikrobiologie, U nemocnice 1200, Praha 6, ČSSR

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The book consists of 13 chapters, of which the first half is devoted to tropical infectious diseases, and the other half concerns pathological conditions caused by life in tropical environment. Apart from cosmopolitan viroses the chapter on viral infections deals with arboviroses: exotic fevers such as chikungunya, o'nyong-nyong, Kyasanur forest disease, Rift Valley fever etc. It also deals with trachoma, virus granulomes, kuru, rickettsial diseases and bartonellosis. In the chapter on diseases caused by bacteria, besides cosmopolitan bacterial diseases also cholera, plague, leprosy and treponematoses (frambesia, pinta, bejel, sodoku) borrelioses etc. are discussed. The next chapter covers cutaneous and systemic mycoses. Out of parasitic diseases the tropical as well as some cosmopolitan protozoonoses and helminthoses are dealt with. The following chapters are devoted to damages inflicted to human health by plant and animal poisons, by humid and hot climate, and are also concerned with nutritional deficiencies. The chapter on anemias includes accounts of hemoglobinopathy, thalassemia, glucose-6-phosphate-dehydrogenase deficiency and nutritional anemia. Attention is also paid to tropical cardiomyopathy, malignant tumors and psychiatric disorders. Last chapters provide accounts of health examinations for persons staying in the tropics and consider tropical infections as occupational diseases. The volume is completed by a list of important monographs and by subject and author indexes.

The Czech and Slovak physicians as well as other specialists go to work in developing countries and vice versa many foreigners visit our country. Air transport has made trips between continents short and has increased the risk of introducing tropical diseases to the countries of temperate zone. The problems of tropical medicine are met with by physicians assessing the consequences of a stay in the tropics while examining the persons who have just returned from such countries and are tackled by medical staff in charge of public health matters and information on preventive medicine. In compiling this volume Dr. Šerý with his co-workers and 17 other top experts have successfully filled in a gap in the Czech medical literature. It is a first text-book on tropical medicine published in the temperate zone country which never possessed colonies and consequently started to study tropical medicine as well as parasitology as late as in the last few decades. The reader may judge for himself that the book is fully up to the standards set by similar publications in countries with a long tradition in tropical medicine. The information accumulated in the book is based on personal knowledge and experience of Czechoslovak specialists. The chapter topics are equally balanced, bibliography is ample and carefully arranged, consisting mostly of titles of manuals on tropical medicine and parasitology. A wealth of original illustrations is an evidence that the authors had their own cameras always ready. It is only to be regretted that reproductions of some photographs are not done on a better paper or in colour.

Dr. J. Jíra, C.Sc.