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PERSISTENCE OF TICK-BORNE ENCEPHALITIS VIRUS IN MONKEYS
VII. SOME FEATURES OF THE IMMUNE RESPONSE

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Summary. — Tick-borne encephalitis (TBE) virus persists in experimentally infected rhesus monkeys in the presence of humoral antibodies. Various dynamics of the humoral response (stable, increasing, decreasing, undulatory titres) have been noted, associated with complete or incomplete set of antibodies. Always present were the virus-neutralizing antibodies, often the complement-fixing antibodies, less frequently precipitating and haemagglutination inhibition (HI) antibodies were found. There was a correlation between the set of antibodies present and the virus-specific antigens expressed; the persisting TBE virus was usually deficient in haemagglutinin synthesis. In cases of asymptomatic infection a more expressed and long-lasting immunity as observed with the persistence of TBE virus in organs of the immune system.

Key words: flavivirus; tick-borne encephalitis; monkeys; persistence; immunity

Introduction

Tick-borne encephalitis (TBE) is a neuroinfection leaving a long-lasting, perhaps a life-long immunity: no reliable cases of repeated infection have been described to date.

The duration of immunity to TBE virus has not been satisfactorily explained yet, little attention has been paid to the possible relationship with the virus persistence. Examination of chronic TBE patients does not offer an unequivocal evidence as to the role of virus persistence due to limited information on the properties and functions of the persisting virus and because of the variability of immunological characteristics. Some workers (Minayeva *et al.*, 1975) noted a decrease of antibody titres during stabilization and regression of the chronic process and elevation of the titres during a progressive course of the disease. Patients suffering from meningoencephalitic and poliomyelitic forms of TBE show enhanced IgM and IgG synthesis and prolonged intervals of their circulation (Kvetkova *et al.*, 1978); these forms are often followed by progressive development of TBE.

Kraminskaya and coworkers (1972) isolated 5 TBE virus strains from the blood and liquor of patients developing hyperkinetic forms of chronic TBE. All these patients showed a humoral response and the titres of virus neutralizing and complement fixing antibodies in their sera were higher than in the cerebrospinal fluid. As shown by Gajdošová and Mayer (1978), mice remain resistant and retain humoral and cellular immunity to asymptomatic Langat virus infection. Although the persisting virus had not been isolated, the authors assumed that a long-term antigenic stimulation might have been caused by defective but antigenically active virus particles.

This paper considers some immunological aspects of TBE virus persistence in rhesus monkeys developing chronic encephalitis and in asymptomatic virus carriers extending the preceding studies dealing with clinical, morphological, virological and pathogenetic features of chronic experimental TBE virus infection (Pogodina *et al.*, 1981*a*, *b*, *c*; Frolova, 1981).

Materials and Methods

Design of persistent infection. 50 rhesus monkeys weighing 2.2—3.2 kg and having no antibodies to the viruses of TBE complex were subjected to intracerebral (i.c.) and subcutaneous (s.c.) inoculations with the TBE virus: strain Vasilchenko (15 animals), strain Aina/1448 (10 monkeys), strain 41/65 (9 monkeys) and mutant Pan 114 No. 1M (6 monkeys). 14 animals developed acute encephalitis, 5 became chronically infected and 31 animals, all of which received s. c. inoculations, were shown to be asymtomatic carriers. Of the latter group, 20 monkeys received 50—100 mg/kg cyclophosphamide one day before and one day after virus inoculation. The strains used and the details of experimental infection have been described elsewhere (Pogodina *et al.*, 1981*a*).

Virological studies. Blood samples were taken from each monkey daily in the course of the first week, then at 3—5 day intervals in the course of 1 1/2 months, and then once in 2—4 weeks. Titres of infectious virus in heparin-treated blood samples were measured by inoculation of 0.03 ml samples into the brain of white mice (5—6 g body mass, 20 animals for each blood sample). Methods of isolation and identification of the persisting TE virus, as well as methods of detection of haemagglutination, complement fixing and precipitating antibodies and the fluorescent antibody technique (FAT) were described previously (Pogodina *et al.*, 1981*a*, *b*, *c*).

Immunological studies. Blood samples were assayed by means of haemagglutination inhibition (HI), complement fixation (CF), diffusion precipitation in agar (DPA) and neutralization tests. HI was carried out according to Clarke and Casals (1958) and DPA was performed as described by Bochkova *et al.* (1974). Neutralization test was run in two modifications: 1) white mice weighing 6—8 g were infected i.c. with a mixture of undiluted serum and a series of 10-fold dilutions of virus (neutralization index); 2) 100 PFU of the virus were mixed with a series of 2-fold serum dilutions and used to infect chicken embryonic fibroblasts; the reduction of the plaque-forming ability served as a measure of virus neutralizing (VN) antibody titre.

The T lymphocyte response was estimated by means of splenocyte migration inhibition test (or a migration inhibition test employing simian inguinal lymph node cells) in the presence of specific antigen (Mayer *et al.*, 1976). The antigen was prepared from the brain of white mice (weighing 4—6 g) after precipitation with protamine sulphate and centrifugation at 15,000 g for 20 min. Antigen prepared from the brain of uninfected mice served as control. The B lymphocyte system was assessed by IgG and IgM synthesis and from the activity of B lymphocytes tested in passive local haemolysis (antibody-forming or plaque-forming cells in suspension) (Larina *et al.*, 1978).

Results

Humoral immunity in acute or chronic encephalitis

The 14 monkeys which developed acute encephalitis had serum antibo dies since days 4 to 6 post-infection (p.i.). At 10—12 days the antibody titre as

Table 1. Types of dynamics of antibody response in monkeys infected s.c. with TBE virus and developing asymptomatic infection

Monkey No.	Virus strain	Day	Antibody titre measured by			
			WN	HI	CF	DPA
8081	41/65	15	2.3 *	0	0	0
		32	2.4	0	0	0
		62	2.8	20	0	0
		75	2.8	20	0	0
		105	3.3	40	0	0
		162	3.2	80	16	4
		216	3.7	320	16	8
		302	2.3	80	0	2
		340	4.5	160	0	0
		368	3.3	80	0	0
8036	41/65	19	2.0	20	ND	ND
		32	1.9	20	0	0
		62	2.0	0	8	0
		92	2.0	0	8	0
		162	1.2	20	0	0
		215	1.4	40	0	0
		277	1.8	20	8	0
		302	1.5	20	8	ND
		340	3.6	80	8	0
		357	3.5	40	8	0
8426	Aina/1448	19	1.4	0	0	4
		25	2.0	10	ND	8
		32	2.6	20	32	4
		62	2.4	0	16	2
		75	2.1	20	8	2
		91	2.6	20	8	0
		102	1.9	20	0	8
8497	Aina/1448	13	1.3	0	0	0
		43	2.5	0	64	0
		99	2.4	0	32	0
		153	2.6	0	8	ND
		176	1.6	0	0	0
8483	Aina/1448	8	1.4	0	0	0
		49	1.4	0	0	ND
		99	2.2	0	0	0
		153	2.3	0	0	0
		215	0.5	0	0	ND
		271	3.3	0	0	0

* Neutralization index values in $\log_{10}$ units. Figures in other columns are the titre reciprocals 0 = negative; ND — not done.

assayed by HI, CF, DPA and neutralization tests was 1 : 320, 1 : 64—1 : 128, 1 : 4—1 : 16 and 3.0 (logarithm of neutralization index), respectively. Five monkeys showed antibody formation in the course of chronic encephalitis. Monkey No. 7478 which was infected i.c. with the attenuated strain Pan 114

Table 2. Humoral immunity induced by different strais of TBE virus

Virus strain	Number of monkeys	Limits (in days) of antibody detection				Features of immunity				
						Antibody dynamics[1]			Antibody set[2]	
		NT	HI	CF	DPA	I	II	III	C	I
46/65	6	1013	340	340	216	3	3	0	2	4
Vasilchenko	11	920	277	700	lg[3]	8	1	2	0	11
Aina/1448	6	307	277	293	75	1	3	2	2	4

[1] — type of antibody dynamics; I — stable level of immunity or an increase at distant intervals; II — cyclic rises and falls of antibody titre; III — decrease after 6—7 months;
[2] — antibody set: C — complete (VN, CF, precipitating and haemagglutinating), I — incomplete (absence of certain antibody type or production of the VN antibody only);
[3] — precipitating antibodies found only in individual sera.

No. 1M developed chronic encephalitis on day 45. No antibodies were found during the first two weeks p.i. On day 14 the antibody titre was 1 : 2 (virus neutralizing antibodies), then increased to 1 : 32 by days 17—21 and to 1 : 512 by day 45. HI antibody titre increased from 1 : 10 (day 17) to 1 : 40 (day 25) and then to 1 : 320 (day 45).

Later on (days 383, 783, 850 p.i.) monkeys with chronic TBE had virus neutralizing antibodies (logarithm of neutralization index between 2.0 and 5.0) and complement fixing antibodies (1 : 32—1 : 64) while the HI antibodies were usually absent. CF and FAT revealed virus-specific antigen in the brain and spinal cord of these animals, and in some cases virulent strains of TBE virus for mice were isolated.

Humoral immunity in asymptomatic infection

Examination of 31 monkeys infected s.c. with the TBE virus allowed to distinguish 3 types of the dynamics of humoral immune response (Table 1): a) decreasing b) stable or increasing at remote intervals; c) undulatory. Differences have been found also regarding the type of antibody induced. Virus neutralizing antibodies were detected regularly while CF and precipitating antibodies as well as HI antibodies were less frequent. Some animals possessed all antibody types, others lacked one or two types or had VN antibodies only. The antibody spectrum was the same in animals that received or did not receive cyclophosphamide.

Immune response to different TBE virus strains

Dynamics of humoral antibodies was compared in 23 monkeys infected s.c. with strains 41/65, Vasilchenko and Aina/1448 in the course of asymptomatic infection lasting 2 1/2 years. Strain 41/65 induced the most prolonged immunity characterized by stable or repeatedly increased titres of antibodies of various types (Table 2). Strain Vasilchenko also induced a long-lasting immunity, but in all of the 11 infected monkeys the antibody set was in-

Table 3. Humoral immunity in cases of virus persistence in the CNS of monkeys

No. of monkey and virus strain	Persistence			Antibody dynamics		
	Observation period*	Localization in CNS	Method of detection	Day of assay	VN	HI
7470 (Pan 114 No. 1M)	45	brain stem	infection of mice	1, 4, 5, 7	0	0
				10, 14, 17	0	0
				21, 25	1 : 2	0
				45	1 : 2	0
6997 (Pan 114 No. 1M)	45	brain stem hemisphere cortex, spinal cord	infection of mice	1, 4, 5, 7	0	0
				10, 14, 17	0	0
				21, 25	1 : 2	0
				35, 40, 45	1 : 2	0
8484 (Vasilchenko)	176	subcortical ganglia spinal cord	co-cultivation, FAT	13	1.8	0
				41	3.2	0
				99	3.7	0
				153	2.4	0
				176	1.2	0

Notice: The VN antibody column shows antibody titres (monkeys 7470, 6997) or $\log_{10}$ values of neutralization index (monkey 8486); the HI column shows antibody titre reciprocals.
* in days

complete. VN antibodies remained at a high level over 900 days, CF antibodies for about 700 days while HI antibodies persisted shorter (227 days). Precipitating antibodies were found only in individual cases. Imunity induced by Aina/1448 was shorter and its dynamics varied.

Immunity in persistent infection of different localization

In some monkeys with asymptomatic persistence of TBE virus, the virus (or antigen) was present in the CNS only, while in others it persisted outside of CNS only or in both the CNS and other organs. In the first case the humoral immunity was usually weak: low antibody titres, rapid disappearance and incomplete antibody sets (Table 3). At an early stage of the infection (day 45), a TBE strain virulent for mice was isolated from the CNS. At later stages the persistence of the virus and viral antigen were found by means of co-cultivation and FAT.

When the persisting virus was localized outside of the CNS (spleen, lymph nodes, liver, kidneys), the immunity was prolonged and the levels of VN and HI antibodies were higher (Table 4). High antibody levels were also observed if virus (antigen) was localized in both the CNS and lymphoid organs. In all of these cases CF and FAT revealed virus-specific antigen in tissues. An infectious virus could be isolated by means of co-cultivation and explantation but not by infecting mice directly. Isolates obtained by the co-cultivation method possessed no haemagglutinating activity. The isolates

Table 4. Humoral immunity in monkeys with extraneural localization of persistent TBE virus

No. of monkey (virus strain)	Characteristics of persistence			Antibody dynamics		
	Observation period (days)	Localization	Method of assay	Day of assay	NT*	HI**
8045 (41/65)	236	spleen, lymph, nodes, kidney	co-cultivation, FAT	15	1.9	0
				25	3.5	20
				62	2.5	0
				76	3.3	160
				113	3.7	160
				162	3.3	40
				216	3.7	80
				236	3.7	20
8493 (Aina/1448)	284	liver	explantation	8	2.4	0
				29	3.2	20
				49	3.3	20
				99	2.0	0
				153	1.7	0
				239	1.0	0
				277	2.6	20
				284	3.8	20
8491 (Vasilchenko)	293	lymphatic nodes, liver spleen, kidney	explantation, FAT	29	3.3	0
				49	4.4	0
				99	2.5	40
				153	2.6	20
				215	2.3	20
				239	3.0	20
				293	3.5	40

* $\log_{10}$ values of the neutralization index.
** Antibody titre reciprocals.

obtained at explantation induced the synthesis of various antigens including haemagglutinin.

Sera of monkeys in which virus persistence was demonstrated both in CNS and inner organs were examined in HI test to assess the dynamics of IgG and IgM synthesis. IgM was found in blood until 76—162 days after single s.c. inoculation in the cases of asymptomatic course. When antibody titres increased at remote intervals after infection, a recurrence of IgM was observed, like in the case of monkey No. 8081 on days 162—216 (Table 1).

The passive local haemolysis test revealed enhanced activity of B lymphocytes in the spleen and lymph nodes of monkeys examined 6 months after s.c. inoculation with strains Vasilchenko and Aina/1448 (2.4—5% plaque forming cells). Four monkeys were examined by means of splenocyte migration inhibition test at 6—9 months after infection. One of them was negative

and three positive. The monkey No. 8483 which had VN antibodies and lacked haemagglutinins (Table 1) showed a 53% inhibition of splenocyte migration. TBE virus was isolated by means of the explantation technique from cerebellum, lymph nodes and liver.

The i.c. infected monkeys showed circulating virus in the blood since days 3—7, the virus titre being $10^{2.5}$—10^{3} LD_{50}/ml. At the time of death of acute encephalitis the titre was $10^{3.9}$—$10^{5.8}$ LD_{50}/ml. In the chronic cases no virus was found in the blood. After s.c. inoculation (asymptomatic infection) the viraemia was cyclic (on days 3—5; 14—17; sometimes days 30 and 45) but usually the blood was free of detectable virus within 21 days.

Discussion

Our results demonstrate that persistence of TBE virus occurs in an immunologically competent organism capable of the B and T cell response. The response may vary, and the variations correlate with clinical and morphological manifestations of the disease, the strain used and the localization of the persisting virus.

Chronic TBE in monkeys develops in the presence of humoral immunity characterized by high titres of VN and CF antibodies, and in either presence or absence of HI antibodies. Some animals showed delayed antibody formation. Using CF test or FAT, a virus specific antigen can regularly be detected in the brain of infected animals. The infectious virus isolated from organs of these animals retains its neurovirulence and cytopatogenic properties in situ or expresses these properties after activation by means of co-cultivation or explantation techniques (Pogodina *et al.*, 1981*c*).

Asymptomatic persistence of the virus also occurs in the presence of humoral antibodies, although the immunity parameters may vary greatly with regard to their dynamics and spectrum. Three types of antibody dynamics have been distinguished (decreasing, stable or increasing and undulatory types). The antibody set may be complete or incomplete. Characteristic features of the response include the invariable presence of VN antibodies, long persistence of CF antibodies, deficient HI antibody synthesis, and prolonged production of IgM (up to 5 1/2 months) or recurrence of IgM in cases of undulatory dynamics of antibodies. The immunity was more expressed when the persisting virus was localized outside of the nervous system (spleen, lymph nodes, liver). Tissues of such animals contain a virus-specific antigen while the infectious virus can be isolated only by means of co-cultivation or tissue explantation. The activated virus can induce synthesis of antigens detectable by FAT, HI or CF tests. Viraemia has not been detected.

Depending on the degree of expression of the viral genome and the immune response, Nathanson and Panitch (1978) classified the persistent viral infections of CNS into 5 groups: 1) the agent persists at high infectious titre and induces no immune response (scrapie); 2) high infectious titre but a weak immune response (lymphocytic choriomeningitis virus); 3) low titre and normal immune response (togaviruses, picornaviruses, visna virus); 4) persistence of viral antigens in combination with a normal or increased response (measles, SSPE);

5) neither persisting infectious virus nor viral antigens can be found in the presence of normal immune response (herpes).

Based on the information on persistence of the Kyasanur Forest disease virus in mice (Price, 1966), togaviruses can be classified into group 3. Classification of the TE virus, as follows from our data, is more complex as characteristics of persistence in primates may vary greatly (Pogodina *et al.*, 1981*c*). Productive infection in the primate tissues is a rare event (5.8% of cases); in the majority of cases the genome persists either expressing some antigens (71.2%) or in a truly latent form (23%). The immunological response, at least of humoral antibodies, may be regarded normal but with certain peculiarities (incomplete antibody spectrum, prolonged IgM circulation). The combination of a normal immunological response with 3 forms of viral genome expression allows to classify the persistent TBE infection in primates into 3 groups according to the above-mentioned classification: into group 3 (persistence of infectious virus in a low titre), group 4 (expression of viral antigens) and group 5 (latent infection). The situation is different with chronic TBE and asymptomatic infection. For chronic encephalitis the group 3 characteristics are typical while asymtomatic infection is rather closer to group 5 and for group 4. The latter circumstance relates TBE to certain paramyxoviruses that persist in the form of incompletely expressed genome. It may be noted that other persisting flaviviruses also have some common properties with agents of groups 4 and 5, e.g. the Modoc virus in hamster (David and Hardy, 1974) or West Nile virus in monkey (Pogodina *et al.*, 1983).

One feature of the humoral immunity accompanying TBE persistence, the absence of some antibody types, usually haemagglutinins, may be due to a charasteristic property of persisting TBE virus, i.e. the deficiency of haemagglutinin synthesis (Pogodina *et al.*, 1981*c*). Another feature, the prolonged IgM circulation, may be indicative of the T lymphocyte defect since the disturbance of the switching of IgM to IgM is, according to Burns *et al.* (1975), a T cell-dependent phenomenon.

Our results confirm the role of virus persistence in maintaining humoral immunity to TBE. In chronic encephalitis, the long antigenic stimulation is provided by viral antigen or the infectious virus persisting in the CNS or in other organs. In cases of asymptomatic infection, the stimulation is mainly due to persisting virus-specific antigens or due to the viral genome which, when activated by some factors, can express viral antigens. The immunity is stronger when the persisting virus (antigen) is localized in organs of the immune system. The latter is in agreement with data of Smorodintsev *et al.* (1975) who suggest that a long-term antibody synthesis is due to the persistence of antigens in lymph nodes (lymphoid follicles and nodular sinuses) and spleen (lymphoid follicles and the marginal zone) as well as due to the proliferation of the stimulated clone of lymphocytes.

The combination of a persisting viral infection and immunity in TBE is a phenomenon that requires further investigation in different aspects (mechanisms of virus maintenance in an immune organism, specific vaccination and persistence, role of the immune response in pathogenesis of chronic TBE).

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