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DEPENDENCE ON THE BIRTH SEASON OF THE ANTIBODY LEVEL AGAINST WEST NILE VIRUS IN THE PAKISTANI POPULATION

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Summary. — Variation of antibody level against West Nile (WN) virus depending on the season of birth was followed among 151 paired serum samples of healthy Pakistani persons in Karachi, collected twice in July and October, 1985. The persons born during the months between February and June had lower positive antibody rate and lower responsiveness in haemagglutination inhibition and neutralizing tests against WN virus than those born during the other months. This phenomenon implies that the ability to produce antibodies against WN virus among Pakistani persons may depend on their birth season.

Key words: Pakistan; West Nile virus; arboviruses; haemagglutination inhibition antibody; neutralizing antibody; season of birth.

Introduction

Previous sero-epidemiological studies in our laboratory have shown that the antibody level against Japanese encephalitis (JE) virus of Japanese population, born during the epidemic years, differed according to their season of birth (Sugamata and Miura, 1985).

To explain the different antibody levels according to the season of birth, the following hypothesis was proposed: The influence of latent infection with JE virus on the immune system may be peculiar at an early stage of life and long-lasting due to a particular grade of maturation of the immune system (Miura *et al.*, 1977; Miura *et al.*, 1982; Sugamata and Miura, 1985). Based on this hypothesis, we assumed that not only the JE virus infection but also other infections with JE-related viruses may have the same birth season effect in other countries outside Japan.

It is well known that West Nile (WN) virus is antigenically related to JE virus, and constitutes the JE complex viruses (Xiao *et al.*, 1986). In Pakistan, it has been reported that WN virus is the most prevalent one among the Pakistani population (Burney, 1966; Burney and Munir, 1966; Hayes and Burney, 1981; Hayes *et al.*, 1982). Therefore, to ascertain the validity of our hypothesis of birth season effect on antibody responsiveness to WN virus,

we investigated the levels of haemagglutination inhibition (HI) and neutralizing (NT) antibodies against WN virus according to the season of birth in 151 paired serum samples of healthy Pakistani persons in Karachi, collected in July and October, 1985.

Materials and Methods

Serum samples were collected twice from the same 151 healthy Pakistani persons; students of primary and secondary schools, medical studients and doctors of a medical college. The first sampling was made between July 28 and August 1, 1985, and the second sampling was made between October 28 and 31 in the same year. The serum samples were stored at −20 °C in Karachi, held on dry ice on the way to Japan, and stored at −70 °C until being assayed for antibodies.

Arboviruses. Paired specimens were tested for the presence of antibodies against WN, JE and Dengue-II (D-II) viruses by NT and/or HI test. A focus reduction neutralization test (Kimura--Kuroda and Yasui, 1985; Kimura-Kuroda and Yasui, 1986) was conducted against WN viruses in cultures of VERO cells grown in Eagle's minimum essential medium (MEM, Nissui Inc.) supplemented with 5 % of foetal bovine serum in 24-well culture plates (NUNC Inc.). Each serum was screened at a 1 : 10 dilution against WN (Egypt−101 strain) virus. In brief, each 120 μl of heat-inactivated (56 °C, 30 min) serum specimen at a 1 : 10 dilution was mixed to the same volume of approximately 100 focus forming units (FFU) of WN virus. After one hr incubation at 37 °C in a CO_2-incubator, 200 μl of the mixture was inoculated onto a sheet of Vero cells, which has been incubated for 24 hr before inoculation. The inoculated cells were incubated for one hr, then the mixture was removed and the cell sheet was covered with 1ml of overlay medium MEM containing 5 % of gamma globulin-free foetal bovine serum (GIBCO Inc) and 0.6 % of methyl cellulose), and it was incubated for two days at 37 °C in a CO_2-incubator. The incubated cell sheet was washed twice with 1ml of MEM, and treated with 0.2 ml/well of antibody mixture, which contains rabbit anti WN virus serum (500 HI units/ml) and complement (10 % of guinea pig serum, Denka Seiken Inc.) in MEM with 25 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethane--sulphonic acid (HEPES, Wako Inc.) (pH 7.3). The treated cell sheet was incubated at 37 °C for two hr, washed twice with 1ml of MEM and stained with 0.3 % trypan blue in 0.01 mol/l phosphate buffered saline (PBS). The foci of infected cells stained with trypan blue were counted under a binocular microscope. For HI tests, JaGAr-01 strain of JE virus, Egypt-101 strain of WN virus and New Guinea strain of dengue-II (D-II) virus were used. The microtiter method for HI test was performed using four haemagglutination (HA) units of antigen of each virus, prepared by the sucrose-acetone extraction method from the infected suckling mouse brain (Clarke & Casals., 1958; Casals, 1967). Each serum specimen was absorbed with goose erythrocytes before testing, and was titrated at two-fold dilutions from 1 : 10. A titre of 1 : 10 or more was recorded as positive.

Measles virus: The microtiter method for HI test (Herrman, 1979) was performed using four HA units of antigen of measles virus (Toshima strain). The serum specimens were treated with kaolin, and absorbed with erythrocytes of *Cercopithecus aethiops* before testing. The specimens were titrated at two-fold dilutions from 1 : 8. A titre of 1 : 8 or more was recorded as positive.

Rubella virus: The microtiter method for HI test (Gershon and Krugman, 1979) was performed using four HA units of antigen of rubella virus (R-1 strain). The serum specimens were treated with kaolin and absorbed with chicken erythrocytes before testing. A titre of 1 : 8 or more was recorded as positive.

Herpes simplex-1 (HS-1) virus: The antigen solid phase enzyme-linked immuno sorbent assay (ELISA) for detecting IgG antibody against herpes simplex-1 virus (HF strain) was performed using the modified method of Voller and Bidwell (1976). Each serum of 1 : 400 dilution with specific ELISA value was recorded as positive.

Entero-70 (E-70) virus: The 50 % plaque reduction neutralization test against E-70 virus (J670/71 strain) was made by the method of Dr. Kawamoto (1979). A titre of 1 : 4 or more was recorded as positive.

Statistical examination: Antibody positive rate and the rate of those with increase or decrease in antibody titer against each virus were calculated for every birth season group. The differences of the rates among those with different season of birth were compared. Significance of the difference was examined by the chi-square test (d.f. = 1).

Table 1. Positive rate of HI and NT antibody against WN virus according to the season of birth of healthy persons in Karachi (collected in July and October, 1985).

Sampling month	Test for antibody	Age (years)	Antibody positive rate by month of birth			
			Total (%)	Feb.—June	July—Jan.	Significance
July	HI	Young (6—20)	38/ 81(47)*	8/30(27)	30/51(59)	$P < 0.01$
		Adult (21—65)	39/ 70(56)	15/29(52)	24/41(59)	$P < 0.5$**
		All (6—65)	77/151(51)	23/59(39)	54/92(59)	$P < 0.02$
	NT	Young	34/ 78(44)	6/28(21)	28/50(56)	$P < 0.005$
		Adult	38/ 72(53)	14/30(47)	24/42(57)	$P < 0.5$**
		All	72/150(48)	20/58(34)	52/92(57)	$P < 0.01$
October	HI	Young	46/ 81(57)	12/30(40)	34/51(67)	$P < 0.02$
		Adult	41/ 70(59)	13/29(45)	28/41(68)	$P < 0.05$
		All	87/151(58)	25/59(42)	62/92(67)	$P < 0.005$
	NT	Young	41/ 78(53)	8/28(29)	33/50(66)	$P < 0.005$
		Adult	39/ 72(54)	11/30(37)	28/42(67)	$P < 0.02$
		All	80/150(53)	19/58(33)	61/92(66)	$P < 0.001$

* No. of antibody positive/No. of tested (%).
** not significant

Results

Antibody positive rate according to the season of birth

About 50% of healthy Pakistani persons living in and around Karachi, had HI and NT antibodies against WN virus regardless whether the sera were sampled in July and/or October, 1985 (Table 1), i.e. there were no significant differences in the positive rates of HI and NT antibodies among samples taken in July and/or October. In addition, when the results were compared between the two age groups, young (6—20 years in age) and adults (21—65 years), the positive rates of HI and NT antibodies in July and October showed no significant differences by age.

Then the antibody positive rates of two age groups were compared according to their month of birth. In the young age group, significantly lower positive rates among the persons born between February and June were observed in both July (HI: $P < 0.01$; NT: $P < 0.005$) and October (HI: $P < < 0.02$; NT: $P < 0.005$) than among the persons born during the other months (July-Jan.). In an adult age group, there were no significant differences of the rates according to their month of birth when tests were made with the serum specimens collected in July. However, the serum specimens collected in October from the same persons showed significantly lower rates among those born between February and June than among those born in the other months (HI: $P < 0.05$; NT: $P < 0.02$). Therefore, in October, those young and adult groups born between February

Table 2. Serum antibody positive rate against measles, rubella, herpes simplex-1 and entero-70 viruses of healthy persons in Karachi (collected in July and October, 1985).

Species of viruses	Sampling month	Age (years)	Antibody positive rate by month of birth			Significance
			Total (%)	Feb.—June	July—Jan.	
Measles	July	Young (6—20)	71/81(88)*	25/30(83)	46/51(90)	$P < 0.5$**
		Adult (21—65)	59/71(83)	25/30(83)	34/41(83)	$P < 0.99$**
	October	Young (6—20)	65/81(80)	22/30(73)	43/51(84)	$P < 0.75$**
		Adult (21—65)	55/71(77)	24/30(80)	31/41(76)	$P < 0.75$**
Rubella	July	Young	71/81(88)	25/30(83)	46/51(90)	$P < 0.5$**
		Adult	63/71(89)	27/30(90)	36/41(88)	$P < 0.9$**
	October	Young	70/81(86)	25/30(83)	45/51(88)	$P < 0.75$**
		Adult	61/71(86)	27/30(90)	34/41(83)	$P < 0.5$**
HS-1	July	Young	55/81(68)	21/30(70)	34/51(67)	$P < 0.9$**
		Adult	43/71(61)	19/30(63)	24/41(59)	$P < 0.75$**
	October	Young	52/81(61)	21/30(83)	31/51(88)	$P < 0.99$**
		Adult	42/71(59)	19/30(63)	23/41(56)	$P < 0.99$**
E-70	July	Young	65/80(81)	25/29(86)	40/51(78)	$P < 0.5$**
		Adult	38/69(55)	15/28(54)	23/41(56)	$P < 0.9$**
	October	Young	61/80(76)	21/29(72)	40/51(78)	$P < 0.75$**
		Adult	37/69(54)	15/28(54)	22/41(54)	$P < 0.99$**

*: No. of antibody positive/No. of tested (%).
HI antibody against measles and rubella, IgG-ELISA antibody against HSV-1 and neutralizing antibody against EV-70 were measured.
** not significant

Table 3. Responsiveness of HI antibody to West Nile virus according to the season birth of healthy persons in Karachi (collected in July and October, 1985).

Response	Age in years	Rate of responsiveness by month of birth			Significance
		Total (%)	Feb.—June	July—Jan.	
Increase[a])	Young (6—20)	22/ 81(27)	6/30(20)	16/51(31)	$P < 0.5$*
	Adult (21—65)	26/ 70(37)	7/29(24)	19/41(46)	$P < 0.1$*
	All	48/151(32)	13/59(22)	35/92(38)	$P < 0.05$
Decrease[b])	Young (6—20)	10/ 38(26)	2/ 8(25)	8/30(27)	$P < 0.95$*
	Adult (21—65)	11/ 39(28)	5/15(33)	6/24(25)	$P < 0.9$*
	All	21/ 77(27)	7/23(30)	14/54(26)	$P < 0.9$*

[a]): No. of positive conversion + No. of antibody increase from July to October/No. of tested.
[b]: No. of negative conversion + No. of antibody decrease from July to October/No. of persons with positive antibody in July.
* not significant

and June showed significantly lower positive rates of HI and NT antibodies than the others.

When the antibody positive rates against measles, rubella, HS-1 and E-70 viruses were compared according to the month of sampling, there were no significant differences between July and October (Table 2). When the results were examined among the two age groups, there were no significant differences in the positive rates against measles, rubella and HS-1 viruses neither in July nor in October. But the antibody positive rate against E-70 virus in the young age group either in July or October was significantly higher than that of adult persons. Among healthy Pakistani persons, about 80% had

Table 4. Responsiveness of NT antibody to West Nile virus according to the season birth of healthy persons in Karachi (collected in July and October, 1985).

Response	Age in years	Rate of responsiveness by month of birth			Significance
		Total (%)	Feb.—June	July—Jan.	
Positive conversion[a])	Young (6—20)	10/44(23)	3/22(14)	7/22(32)	$P < 0.5$*
	Adult (21—65)	10/34(29)	3/16(19)	7/18(39)	$P < 0.5$*
	All	20/78(26)	6/38(16)	14/40(35)	$P < 0.1$*
Negative conversion[b])	Young (6—20)	1/34(3)	0/ 6(0)	1/28(4)	$P < 0.5$*
	Adult (21—65)	9/38(24)	5/14(36)	4/24(17)	$P < 0.5$*
	All	10/72(14)	5/20(25)	5/52(10)	$P < 0.1$*

[a]): No. of positive conversion from July to October/No. of persons with negative antibody at July.
[b]): No. of negative conversion from July to October/No. of persons with positive antibody in July.
* not significant

Table 5. Responsiveness to measles, rubella, herpes simplex-1 and entero-70 viruses according to the season of birth of healthy persons in Karachi (collected in July and October in the year of 1985).

Species of viruses	Response	Age in years	Rate of responsiveness by month of birth			
			Total(%)	Feb.—June	July—Jan.	Significance
Measles	Increase[a])	Young (6—20)	17/81(21)	4/30(13)	13/51(25)	$P < 0.2$*
		Adult (21—65)	24/71(34)	8/30(27)	16/41(39)	$P < 0.5$*
	Decrease[b])	Young (6—20)	18/71(25)	6/25(24)	12/46(26)	$P < 0.9$*
		Adult (21—65)	20/59(34)	9/25(36)	11/34(32)	$P < 0.9$*
Rubella	Increase	Young	10/81(12)	5/30(17)	5/51(10)	$P < 0.5$*
		Adult	15/71(21)	6/30(20)	9/41(22)	$P < 0.9$*
	Decrase	Young	17/71(24)	8/25(32)	9/46(20)	$P < 0.25$*
		Adult	15/63(24)	6/27(22)	9/36(25)	$P < 0.9$*
HS-I	Increase	Young	4/81(5)	2/30(7)	2/51(4)	$P < 0.5$*
		Adult	19/71(27)	8/30(27)	11/41(27)	$P < 0.75$*
	Decrease	Young	13/55(24)	6/21(29)	7/34(21)	$P < 0.5$*
		Adult	16/43(37)	8/19(42)	8/24(33)	$P < 0.75$*
E-70	Increase	Young	15/80(19)	6/30(20)	9/50(18)	$P < 0.75$*
		Adult	15/69(22)	4/28(14)	11/41(27)	$P < 0.25$*
	Decrease	Young	33/65(51)	13/25(52)	20/40(50)	$P < 0.9$*
		Adult	22/38(58)	10/15(67)	12/23(52)	$P < 0.5$*

[a]): No. of positive conversion + No. of antibody increase from July to October/No. of tested.
[b]): No. of negative conversion + No. of antibody decrease from July to October/No. of persons with positive antibody in July.
* not significant

antibodies against measles and rubella viruses, and about 60% against HS-1 and E-70 viruses. The positive rate against each of those viruses, however, did not differ significantly when compared according to their month of birth.

The rate of antibody responsiveness according to the season of birth

The rate of those who increased or decreased in HI antibody titre was examined among the paired serum samples collected twice in July and October (Table 3). Thirty two per cent of all the persons showed positive conversion or increase of HI antibody, and 27% of the persons with positive HI antibody in July showed negative conversion or decrease of HI antibody in October. When the rate of the increase or decrease in HI antibody titre was examined according to the month of birth of corresponding persons, a significantly lower rate with increased HI antibody titre was observed among Feb.—June born persons ($P < 0.05$) than among others. But there were no significant differences in the rate of decreased HI antibody according to their month of birth. Comparing the rate of increased antibody titre among the two age groups, both young and adult persons born in Feb.—June showed

a lower rate than the others. In NT antibody (Table 4), the lower rate of the positive antibody conversion was also observed among the persons born in Feb.—June. The rate of negative conversion of NT antibody in persons born between Feb.—June was a little higher than of the others.

The rates of increased or decreased antibody titre against measles, rubella, HS-1 and E-70 viruses were also compared according to the month of birth (Table 5). There were no significant differences among these four viruses.

Discussion

Previous epidemiological studies in our laboratory have shown that the incidence rate of Japanese encephalitis and the protective efficacy of JE vaccine among Japanese population differed according to their season of birth (Miura *et al.*, 1976; Miura *et al.*, 1977). These phenomena suggested that the degree of resistance to JE virus infection of Japanese population differs according to their season of birth. It was also revealed that the Japanese population born during the epidemic season (July—Oct.) of JE epidemic years showed lower HI antibody positive rate against JE virus than those born during the non-epidemic season (Nov.—June) of the same year (Sugamata and Miura, 1985). These studies were made to confirm the hypothesis that the influence of latent JE virus infection on the immune system may differ at early stages of life probably due to a different grade of maturation of the immune system, and this could explain the different susceptibility of human population according to the season of birth to JE virus infection, which shows a seasonal epidemic (Miura *et al.*, 1977; Miura *et al.*, 1982; Sugamata and Miura, 1985).

Based on these evidences, we assumed that not only JE virus but also other antigenically related JE complex viruses may have the same birth season effect in other countries besides Japan, since JE complex viruses, or some mosquito-borne arboviruses have the same epidemiological characteristics as follows: (1) the infections of the mosquito-borne arboviruses show seasonal epidemics annually, (2) major mode of infection with arboviruses is a latent infection, (3) the latent infection may occur also during the foetal stage.

In Pakistan, it has been reported that the WN, D-II and some other flaviviruses exist (Hayes and Burney, 1981). However, it is considered that WN virus is the major one (Burney, 1966; Burney Munir, 1966; Hayes *et al.*, 1982). By now, the existence of JE virus has never been confirmed in Pakistan, while severe epidemics of JE have been reported from the neighbouring country, India (Umenai *et al.*, 1985). Only recently, some neurological patients were suspected to be JE cases by clinical and serological findings (Sugamata *et al.*, 1986; Takasu *et al.*, 1986). Therefore, we studied initially the HI antibody levels according to the season of birth against JE, WN and D-II viruses, and detected the lower HI antibody positive rate against the three viruses in Pakistani persons born between Feb.—June than in those born during the other months. The results indicate that the differences

of the levels of antibodies among Pakistani population according to the season of birth are caused by infection with antigenically related JE complex virus, probably by the WN virus.

The positive rate of HI and NT antibodies against WN virus was distinctly different when they were compared between the two groups differing by their season of birth. The young (6—20 years in age) and adult (21—65 years) persons born between February and June showed lower levels of antibody to WN virus than those born during the other months. For the other viruses besides WN virus, there were no significant differences of the levels of antibodies according to the season of birth. Moreover, the different responsiveness of antibody production was observed as an increase or decrease in titre of HI and NT antibodies between the paired serum samples collected in July and October. The rate of persons with increased antibody titre was lower, and the rate of those with decreased antibody titre was higher among the persons born in Feb.—June. Therefore, it is considered that the lower levels of the antibodies against WN virus among Feb.—June born persons is due to the lower ability to produce antibody against WN virus and also the feebleness to maintain it.

The lower levels of antibodies against WN virus among Feb.—June born persons were observed in ages from 6 to 65 years. This implies that the influence of the birth season effect of latent infection at an early stage of life with WN virus on the ability to produce HI and NT antibodies in later life must continue for long years. This long-lasting birth season effect has been reported in Japanese population against JE virus (Miura *et al.*, 1976; Miura *et al.*, 1977; Sugamata and Miura, 1985), and in this study, the same phenomenon was observed in Pakistani population against WN virus. As an explanation of the birth season effect by JE virus infection, different grades of resistance to JE virus infection according to the different foetal stages of preliminary JE virus infection has been shown in mice to last up to 180 days after birth (Miura *et al.*, 1982). This difference in grade of resistance seems to correlate with the difference in frequency of vertical JE virus infection according to the different foetal stages on which JE virus infection was made in mice (Sugamata and Miura, 1982).

Among the Japanese population, those born during the epidemic season (July—Oct.) of JE epidemic years showed lower level of HI antibody against JE virus than those born during non-epidemic season (Nov.—June) of the same years (Sugamata and Miura, 1985). Therefore, the same mechanism as in the latent JE virus infection among the Japanese population can be applied to the WN virus infection among the Pakistani population in Karachi area, the epidemic season of WN virus in Karachi area may occur in the months between February and June.

In the same persons, viral antibodies were measured against measles, rubella, HS-1 and E-70 viruses. No significant differences were observed according to the season of birth against all the four viruses. The seasonality of epidemics, however, is known in connection with measles and rubella viruses in most countries. Kimura *et al.*, (1983) described different antibody

levels against measles and rubella viruses among Japanese population according to the season of birth. It is also known that infection with E-70 virus is more prevalent in the summer in subtropical countries (Hung and Kono, 1979). One possible explanation for this difference is that there are all year round epidemics of infections with viruses such as measles, rubella, HS-1 and E-70 in the Karachi area. Another possibility is that the virological characteristics of the JE complex viruses may be different from rubella, HS-1 and E-70 viruses as to induction of specific long-lasting immunity when infected at a foetal stage.

The long-lasting birth season effect on the immune ability to the later WN virus infection among Pakistani population presented in this paper is similar as have been shown in JE virus infection among Japanese population. For elucidation of the mechanism of long-lasting birth season effect on the immune responsiveness of humans against JE complex viruses, another studies are required about the influences of latent infections with JE complex viruses in the foetal and perinatal life.

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