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A Seroepidemiological Study of *Toxoplasma* Infection in Nagasaki by ELISA

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Abstract

Serum specimens from 160 and 286 apparent healthy inhabitants aged from 20 to 79 years residing in Nagasaki city and in Nakadori Island, respectively, were examined to measure specific IgG antibodies to *T. gondii* by ELISA. It was found that the overall positive rate of toxoplasma infection was significantly higher in Nakadori Island (57.7%) than in Nagasaki city (46.3%). The peak level of positive rate was seen in the age of 60–69 years in Nakadori Island and in 70–79 years in Nagasaki city. The frequency of high antibody titer carriers was higher in Nakadori Island than in Nagasaki city. Furthermore, annual new infection risk was higher at the age of 35 to 45 years (5.7%) in Nagasaki city and at the age of 45 to 55 years (8.2%) in Nakadori Island. Discrepancies of the positive rate, antibody levels and infection rates between two regions might be attributed not only to the transmission routes but also to the host factors.

Key words: *Toxoplasma infection, Enzyme-linked immuno sorbentassay, Seroepidemiology, Nagasaki.*

Introduction

It has already been reported that the positive rate for *Toxoplasma* infection by serological tests differs from place to place (Feldman and Miller, 1956; Tizard *et al.*, 1977; Suzuki *et al.*, 1985) and increases with age (Tizard *et al.*, 1977; Van der Veen and Polak, 1980). The infection remains asymptomatic in many cases (Feldman, 1974) and is occasionally reactivated in compromised host (Ruskin and Remington, 1976; Velimirovic, 1984). Furthermore, diagnosis of such reactivated infection is frequently difficult. In our department we recently experienced brain abscess caused by reactivation of *Toxoplasma* infection during a combination therapy of corticosteroid and anticancer drugs for a patient with malignant thymoma, but the reactivation of *Toxoplasma* infection was not diagnosed until the patient died (Uzuka *et al.*, 1982). Prevalence of *Toxoplasma* infection in wide ranges of inhabitants in Nagasaki prefecture has not been investigated for a long period for since Murakami (1964) reported their epidemiological study result on toxoplasmosis among residents. Following to our study on the effect of *Toxoplasma* infection in pregnant women (Matsumoto *et al.*, 1982; Suzuki *et al.*, 1983), the present study aimed at epidemiological survey of *Toxoplasma* infection in Nagasaki prefecture as a step for analysis of the transmission routes of *T. gondii* in this region, although *Toxoplasma* infection is presumed to be due to ingestion of either oocysts or tissue cysts containing bradyzoites (Remington and Mcleod, 1986).

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Materials and Methods

Serum samples. During a period from October to December 1984, blood samples were collected from apparently healthy inhabitants of Nagasaki city (urban area) and an island in Nagasaki prefecture, Nakadori Island (rural area). The number of serum samples randomly selected from the above for tests were 160 in Nagasaki city and 286 in Nakadori Island.

The subjects aged from 20 to 79 years and the sex ratio of male to female was 1:3. The sera were stored at −20°C until use.

Control serum. A serum with an antibody titer of 1:256 by the dye test modified by Kobayashi *et al.* (1968) was used as positive control. Negative control serum *T. gondii* was prepared by the absorption method. Briefly, the serum with negative dye test was incubated with purified *T. gondii* (Bodner *et al.*, 1972) at 37°C for 1 hr and then at 5°C for 24 hr. The serum was then centrifuged at 6,000 rpm at 5°C for 30 min. The supernatant was filtered through a membrane filter with a pore size of 0.45 μm, and was stored at −20°C until use.

ELISA procedure. For the determination of IgG antibody to *T. gondii,* the micro-ELISA technique by Voller *et al.* (1976) was used with some modification. The test was performed as described below: Antigen (Bodner *et al.*, 1972) was diluted in 0.05 M carbonate buffer at pH 9.6. After pouring 100 μl of diluted antigen into the wells of a flat-bottomed microelisa plate (Immulon of Dynatech Microelisa System), the microtiterplate was kept at 5°C over night. The plate was washed with phosphate buffered saline (PBS)-Tween 20 for 3 min. This process was repeated three times. The plate was then shaken to dry and used soon or stored at −20°C until use. 100 μl of the test serum diluted in PBS-Tween 20 containing 0.6% bovine serum albumin was added into the wells and the plate was incubated at 37°C for 1 hr in a humid chamber. After the incubation, the plate was washed with PBS-Tween 20 three times. Then, 100 μl of alkaline phosphatase-labeled goat IgG fractions against human immunoglobulins (Tago Inc., Surlingame, CA., USA), diluted to 1:1,000 with PBS-Tween 20, was added into each well and the plate was incubated again at 37°C for 1 hr in a humid chamber. After the incubation, the washing with PBS-Tween 20 was repeated as described above. 100 μl of substrate solution prepared by adding 1 mg of p-nitrophenylphosphate to 1 ml diethanolamine buffer (pH 9.8) was added to each well and the plate was incubated at room temperature for 30 min. After this step the absorbance of each well was read by Microelisa Auto Reader (Dynatech Instruments Inc., Santa Monica, CA., USA). The appropriate concentration of antigen was determined by the checkerboard titration using positive and negative sera. It was finally decided to use the antigen of 0.4 μg/100 μl protein in a well (Lowry *et al.*, 1951).

Antibody level. Two fold serial dilutions of serum samples (beginning with a dilution of 1:20) were prepared using PBS-Tween 20 containing 0.6% bovine serum albumin. Optical density values greater than 3 times that of the negative control for the corresponding dilution were considered to be significant. The end point of titration was expressed by the reciprocal of the highest dilution. Sera with antibody titers equal or more than 1:1,280 were categorized as having high titer.

Annual new infection risk (k). The risk of seronegative persons to acquire *Toxoplasma* infection, that represents a percentage of persons acquiring infection per annum per 100 seronegative persons, was calculated by the following formula.

$$k = \frac{\log_e P_0 - \log_e P_1}{t} \times 100,$$

where P_0 is the percentage of negative sera in a younger age group, P_1 that in an older age group, e is the base of natural logarithm and t is the interval (years) between the median ages of both age groups (Van der Veen and Polak, 1980).

Statistical calculation. In order to check statistic difference, chi-square test and Student's t-test were adopted.

Results

Positive rate.

As shown in Table 1, overall positive rate in ELISA of all the inhabitants was significantly higher in Nakadori Island (57.7%) than in Nagasaki city (46.3%). The positive rate attained to peak level in 70–79 year-old group in the city, while in the island the peak was

Table 1 Result of ELISA assay detecting specific IgG antibody to *Toxoplasma gondii* in healthy inhabitants living in Nagasaki city and Nakadori Island in Nagasaki prefecture

Age in years	Areas	No. of samples examined	No. of positive samples (%)*	Antibody level in positive samples (mean±S. D.)†	No. of samples (%) with high titer (≧1280)*
20-29	N. city[1)]	40	10(25.0)	2.68±0.45	4(10.0)
	N. Island[2)]	40	5(12.5)	2.38±0.62	1(5.0)
30-39	N. city	40	8(20.0) §‡	2.76±0.52	2(5.0)
	N. Island	40	17(42.5)	2.34±0.48 §‡	1(2.5)
40-49	N. city	40	22(55.0) ‡	2.16±0.69 §‡	5(12.5)
	N. Island	38	16(42.1)	3.18±0.51	10(26.3)
50-59	N. city	40	20(50.0) §‡	2.16±0.67 §‡	4(10.0) ‡
	N. Island	71	53(74.6)	3.11±0.59	37(52.1)
60-69	N. city	40	24(60.0) §‡	2.51±0.59 §‡	5(12.5) ‡
	N. Island	49	39(79.6)	3.13±0.54	25(51.0)
70-79	N. city	40	27(67.5) §‡	2.57±0.58 §‡	6(15.1) ‡
	N. Island	48	35(72.9)	3.21±0.36	27(56.3)
Total	N. city	160	111(46.3) §‡	2.42±0.63 ‡	26(10.8) ‡
	N. Island	286	165(57.7)	3.05±0.58	101(35.3)

1): Nagasaki city, 2): Nakadori Island, *: Statistical analysis was done by chi-square, †: After logarithmic conversion of data, statistical analysis was performed by t-test. ‡: $p<0.01$, §: $p<0.05$.

seen in 60–69 years old. In the city the positive rate was significantly higher in persons over 40 years of age than those below 39 years. On the other hand, in the island the positive rates in age groups of 50's and more were significantly higher than in lower age groups. In comparison with the positive rate among age groups of the city and island, significantly higher positive rates were shown in the 50's and 60's in the island. As shown in Fig. 1 the increase in the positive rate with age in the city and island was 0.9 and 1.3% per year as calculated by the linear regression from the means at ages of 25 and 75 years, respectively. Correlation of coefficient of the two linear regressions was 0.9 each.

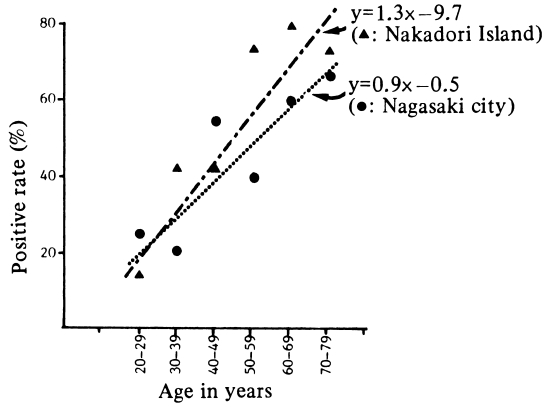

Fig. 1 Positive rate in ELISA detecting IgG antibodies against *Toxoplasma gondii* by age groups in two populations.

Frequency distribution of titers.

Frequency distribution of antibody titers in samples from each age group was shown in Fig. 2. In the city, the frequency curves in the 20's and 30's were of bimodal pattern showing clearly the presence of two subpopulations, negative and moderately high or high titered sera. The titer that ranged from 1:20 to 1:40 was detected much frequently at the age of 40 years or more than those below 39 years old. In the island the distribution in the 20's, 30's, 40's and especially at 70's showed bimodal pattern. As shown in Table 1, frequency of hightitered samples was higher in the island than in the city, especially in those of 50 and more years.

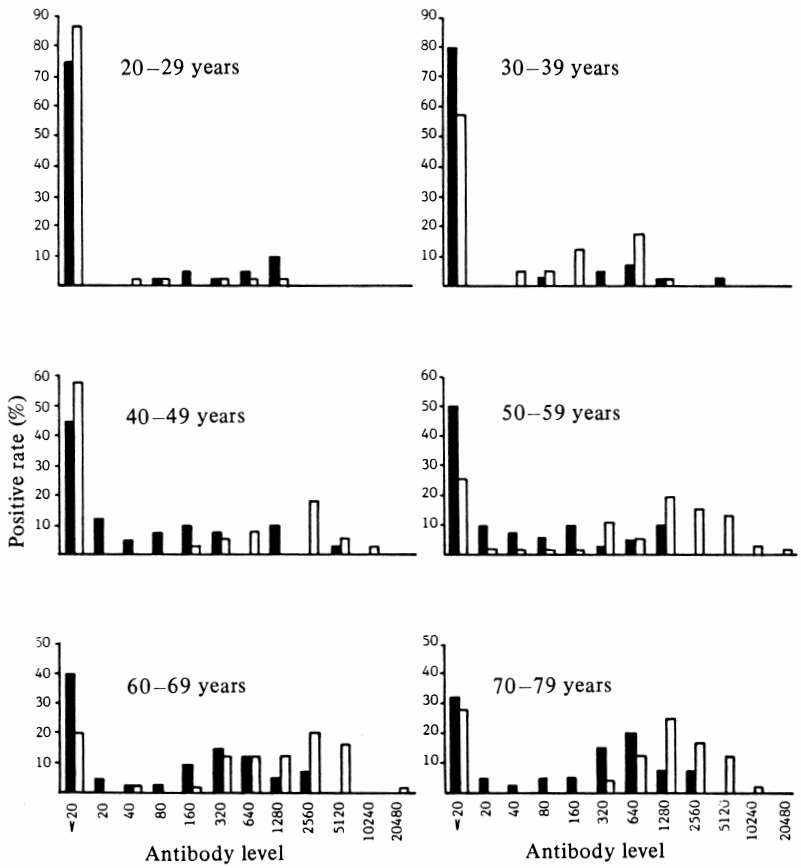

Fig. 2 Frequency distribution of antibodies to *Toxoplasma gondii* at different age groups in Nagasaki city and Nakadori Island in Nagasaki prefecture.
■: Nagasaki city, □: Nakadori Island.

Antibody level.

The level of antibody to *T. gondii* in positive samples was shown in Table 1. The average antibody level of all the inhabitants in the island was significantly higher than that in Nagasaki city. The antibody level of the same age group between two areas revealed that those of 40 and more years old were significantly higher in the island than in the city. In Nagasaki city the levels of antibody of those between 40 and 59 years old were significantly lower than those in the other age groups. In the island the levels of antibody at 40 or more years old were significantly higher than those below 39 years of age.

Infection risk by age.

In Nagasaki City, the highest annual new infection risk of 5.7% was found at ages of 35–45 years (Table 2). After the age of 55 years, the rate was relatively low, 2.2 and 2.1%. On the other hand, the highest infection risk, 8.2%, in Nakadori Island was detected in the age group, 45–55 years, while the risk in the age group of 25–35 years, was 4.2%.

Discussion

As a first step of seroepidemiological survey of *T. gondii* infections in Nagasaki prefecture: Nagasaki city and Nakadori Island were chosen because of the differences in human ecology and environmental conditions. Major occupations of inhabitants in Nagasaki city are business and laborers in various offices, enterprises

Table 2 Frequency of negative sera in each age group and annual infection risks between median ages of successive age groups in Nagasaki city and Nakadori Island in Nagasaki prefecture

Age group (years)	Areas	No. of samples	No. of negative samples (%)	Median age groups (years)	Annual infection risks (%) in *	
					N. city	N. Island
20-29	N. city[1)]	40	30(75.0)			
	N. Island[2)]	40	35(87.5)			
				25-35	−0.6	4.2
30-39	N. city	40	32(80.0)			
	N. Island	40	23(57.5)			
				35-45	5.7	−0.1
40-49	N. city	40	18(45.0)			
	N. Island	38	22(57.9)			
				45-55	−1.1	8.2
50-59	N. city	40	20(50.0)			
	N. Island	71	18(25.4)			
				55-65	2.2	2.1
60-69	N. city	40	16(40.0)			
	N. Island	49	10(20.4)			
				65-75	2.0	−2.8
70-79	N. city	40	13(32.5)			
	N. Island	48	13(27.1)			

*: See the formula shown in Materials and Methods.
1): Nagasaki city. 2): Nakadori Island.

and industries. On the other hand, those in Nakadori Island are fishing and farming. From the environmental view, there are more farm fields and bushes in the island than in the city. Prior to present study, ELISA, dye test and indirect haemagglutination test (IHA) methods were compared. Regression coefficient of result between the dye test and ELISA was 0.92 and that between the IHA and ELISA was 0.92. However there was slight discrepancy in low antibody level which indicates that ELISA positivity was a little higher than other methods (Pallangyo *et al.*, 1985). Hence, ELISA method was used in this study. ELISA tests for *T. gondii* showed higher rate in Nakadori Island (57.7%) than in Nagasaki city (46.3%). Tizard *et al.* (1977) have previously reported that antibodies to *T. gondii* are more prevalent in small cities than in large ones, suggesting more infections in rural areas than in cities. However, a previous positive rate of inhabitants in Nagasaki city more than 20 years old by dye test in 1964 was 12.6% (Murakami, 1964). This was much lower than the present positive rate. Furthermore, positive rate at the same age groups in Hyogo prefecture (18.5%) (Takahashi *et al.*, 1985) was lower than that of ours. With regard to the crucial factors which modulate *Toxoplasma* infection, Tizard *et al.* (1976, 1977) have pointed out importance of apparent cycles in rodents or wild birds that could be readily and rapidly transmitted to the human population through cats.

In Nagasaki city and Nakadori Island the positive rate in ELISA increased with ages. This phenomenon was also reported by other researchers (Tizard *et al.*, 1977; Van der Veen and Polack, 1980). Furthermore, in our study positive rate increased at the rate of 0.9% per year in Nagasaki city and 1.3% in Nakadori Island between 20 to 79 years old. These

annual incidences were similar to that in Paris (0.9%) (Feldman, 1974), lower than that in El Salvador (5.5%) (Remington *et al.*, 1970) and in Ontario (1.7%) (Tizard *et al.*, 1977), but were higher than those in New York city (0.1%) and Ohio (0.2%) (Feldman, 1974).

The positive rate by age attained to peak level at slightly younger age group in Nakadori Island (60's years) than in Nagasaki city (70's years). The positive rates at 50's and 60's age groups were significantly higher in the island than in the city, but were similar at 70's. These results suggested that the positive rate might attain to the similar level at older ages during human life in limited area.

The antibody level and the positive rate of inhabitants in the island was significantly higher than in the city. The frequency distribution of high-titer carrier increased with age in the island, while that of the city was almost similar at each age group. Some researchers reported that the frequency distribution of high-titer carrier gradually decreased with age (De Roever-Bonnet *et al.*, 1980) or was detected at younger age group (15–19 years old) (Van der Veen and Polack, 1980). The reason for difference of the antibody level at different age groups is not yet clear. However, a possibility for this in Nakadori Island may be that they are not only exposed frequently to either living *T. gondii* or antigens of this parasite but also provided with characteristic feature in modulated cellular immunity. Because it is described that positive rate for adult T-cell leukemia virus in inhabitants of Goto Islands where Nakadori Island belongs is significantly higher than that in any other place of Japan and the positive rate increase with ages (Kinoshita *et al.*, 1985).

Low antibody titer carriers were detected more frequently in old inhabitants in Nagasaki city and at the age from 50 to 59 years old in Nakadori Island. In acute symptomatic toxoplasmosis high level of antibody to *T. gondii* is produced (Welch *et al.*, 1980; Iida *et al.*, 1981), while it is unknown whether the low antibody titer implicates primary infection in the form of asymptomatic infection or not.

From the results of the present study the infection risk of seronegative persons appeared to be higher at the age of 35 to 45 years (5.75%) in Nagasaki city and at the age of 45 to 55 years (8.24%) and 25 to 35 years (4.20%) in Nakadori Island. Van der Veen and Polack (1980) have reported that the highest risk (approximately 3%) was found at the age of 12.5 to 25 years which might have been caused by the popular out-of-doors consumption of snacks by people in that age group. However, in our study it is unknown why the infection risk was high at the above mentioned age and differed between Nagasaki city and Nakadori Island. To clarify the discrepancy, not only the transmission routes but also the host factors that relate to cellular immunity should be analyzed.

In the present study difference of positive rate and antibody level in *T. gondii* infection were detected between Nagasaki city and Nakadori Island in Nagasaki prefecture. But the routes by which these inhabitants acquired infection are not well known. Apart from the congenital route of infection two major routes are believed to exist; ingestion of tissue cysts through raw or partially cooked meat and sporulated/oocysts in foods and flies or cockroaches (Remington and Mcleod, 1986). To analyze the actual transmission route it should be necessary to detect antibodies against unique stage-specific oocyst/sporozoite antigens in inhabitants in these areas (Kasper *et al.*, 1984; Kasper and Ware, 1985).

Further investigation will be necessary to examine various populations from different places in order to clarify the transmission of toxoplasmosis in Nagasaki prefecture.

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