The Role of Cell Mediated Immunity in Coxsackie B Viral Myocarditis

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The role of cell mediated immunity (CMI) in the pathogenesis of coxsackie B (Cox.B) viral myocarditis in the adult were immunologically investigated. The number of types of neutralizing antibody in patients with Cox.B viral myocarditis was more than that in controls. This fact suggested that these patients had a history of previous Cox.B viral infections. In the patient with Cox.B viral myocarditis, neutralizing antibody titer was increased as 20 folds by the reinfection. And also macrophage migration inhibition test showed that CMI was enhanced not only against the same type but also against the other types of Cox.B group viruses. In conclusion, it may be essential in the occurrence of adult myocarditis that the patient has been infected by Cox.B virus and immunized against the other types as well as the same type of Cox.B group viruses. CMI may also play a critical role in the occurrence of Cox.B viral myocarditis.

A large number of viruses are well-known to produce myocarditis.¹⁻⁴ Of all viruses, the coxsackie B (Cox.B) viruses have been most firmly linked with clinically evident myocarditis. The mechanism of viral myocarditis in the adult would be fundamentally different from that in the newborn.⁵

Namely, newborn myocarditis may be produced by the direct invasion of the virus into myocytes. On the contrary, adult myocarditis may be produced by allergic or autoimmune reaction against an initial infection.

The aim of this study is to prove immunologically this hypothesis by animal experiments and clinical observations.

Key Words:

Viral myocarditis
Coxsackie B group viruses
Serum neutralizing antibody titer
Cell mediated immunity
Macrophage migration inhibition test

SUBJECTS AND METHODS

Cox.B viral myocarditis was diagnosed by the combination of clinical symptoms (included chest X-ray and ECG findings) and serum neutralizing antibody titer (NAT). Serum and lymphocytes were populated from these patients and served for this study.

Cox.B Neutralization Test (NT)

Roller tube tissue cultures of Hela were used for this study. Serum (0.6 ml) was added to the Puck's solutions (5.4 ml) and inactivated in a 56°C water bath for 30 min. Six ml of the 10% diluted solution were followed by serially four-fold dilutions. The equal volume of Cox.B virus solution (100 TCD₅₀/0.1 ml) were added to each serum dilution. The mixtures were incubated at 37°C for 60 min, and 0.2 ml of them were inoculated into 4 culture tubes each.

Cell controls and virus back titrations were performed. NAT was indicated as 50% neutrali-

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TABLE I CHANGES OF NEUTRALIZING ANTIBODY TITERS IN ADULT MYOCARDITIS

Case 1. S.T. 77 y.o. male

Days after the onset of symptom	CB1	CB2	CB3	CB4	CB5	CB6
25	20	1280	20	20	< 5	< 5
90	20	240	20	20	< 5	< 5

Case 2. U.T. 26 y.o. male

Days after the onset of symptom	CB1	CB2	CB3	CB4	CB5	CB6
14	80	1280	< 5	320	< 5	20
82	80	320	< 5	320	< 5	20
117	40	80	< 5	80	< 5	20

CB = Coxsackie B virus

TABLE II DISTRIBUTIONS OF POSITIVE AND NEGATIVE RESPONSES OF NEUTRALIZING ANTIBODY (NA) AGAINST EACH TYPE OF COX.B₁₋₆ VIRUSES

	The number of positive and negative responses of NA in Cox.B ₁₋₆						Total (No. of Cases)
	Negative	Positive					
		1	2	3	4	5	
Normal Controls	5 (8%)	21 (35%)	18 (30%)	9 (15%)	6 (10%)	1 (2%)	60
Heart disease without myocarditis	1 (7%)	5 (36%)	5 (36%)	0	3 (21%)	0	14
Myocarditis	0	3 (11%)	14 (52%)	8 (30%)	2 (7%)	0	27

zation point. Titers to each individual Cox. B₁₋₆ viruses were also determined.

Macrophage Migration Inhibition Test (MIT)

Experimental technique was improved over previous methods^{6,7}: the cover glass method⁸ and a new simple macrophage migration technique modified from Kikuchi's procedures⁹ were used in this study.

Macrophage inhibition assay. Small capillary tubes sealed at one end were filled with the cell suspension, which was the mixture of guinea-pig macrophages from the peritoneal exudate cells of non-sensitized guinea-pigs and human peripheral blood lymphocytes from the patients with Cox.B viral myocarditis. The capillary tubes were centrifuged at 900 rpm for 5 min. The capillaries were cut at the cell debris interphase. The portions containing the cells were placed in chambers. The chamber consisted of 2 pieces of cover

glass (24 × 24 mm) and 4 capillaries containing the cells. Four capillaries were held at a corner of cover glass by a small amount of silicone (Dow Corning). The chambers were filled with antigen solution (UV-inactivated Cox. B group viruses) and incubated at 37°C for 20 hours in CO₂ incubator. The area of migration was measured by projecting the microscopic image of cell migration from each capillary onto drawing paper with a projecting prism. The outline of this migration was drawn and then measured by planimetry. Instead of antigen, Eagle MEM medium was used as the control. The following formula was used to express the extent of migration of cells (macrophage cells):

migration rate (%) = (average area of migration with antigen) / (average area of migration without antigen) × 100

The average area was calculated with at least 8 capillary tubes. The values of less than 80% of

Fig.1. The distribution of NAT against Cox.B group viruses.

migration rate were judged as positive.

RESULTS

Changes of Neutralizing Antibody Titers (NAT) in 2 Adult Cases with Cocksackie B Viral Myocarditis

Table I shows changes of NAT in typical Cox. B viral myocarditis. As prominent changes of NAT were seen in Cox.B₂ virus, the patients were diagnosed as Cox.B₂ viral myocarditis. An increase of neutralizing antibody (NA) against types B₁, B₃ and B₄ was also observed in Case 1. Similarly, a significant increase of NA against types B₁, B₄ and B₆ as well as B₂ was demonstrated in Case 2. These findings suggest that

both cases have been affected by a few types of Cox.B viruses.

Comparison of the Number of Types of NA and Its Titers against Cox.B Group Viruses in Patients with Cox.B Viral Myocarditis and Heart Diseases without Myocarditis, and also in Normal Controls

The number of types of NA and its titers against Cox.B group viruses was assessed in 27 patients with Cox.B viral myocarditis, 14 patients with heart diseases except myocarditis and 60 normal controls. Table II shows the distribution of positive and negative responses of NA against Cox.B group viruses. Approximately one third of normal controls and patients with heart diseases except myocarditis had one or two types

TABLE III CHANGES OF NAT AND MIT IN 2 TYPICAL EXPERIMENTS

Exp. 1 Guinea pig A							Exp. 2 Guinea pig B																																		
Cox.B ₃ virus + Complete Adjuvant into footpad							Cox.B ₃ virus + Complete Adjuvant into footpad																																		
↓	<table><tr><td>27 days*</td><td>B1</td><td>B2</td><td>B3</td><td>B4</td><td>B5</td><td>B6</td></tr><tr><td>MIT</td><td>98</td><td>101</td><td>104</td><td>101</td><td>113</td><td>102</td></tr></table>						27 days*	B1	B2	B3	B4	B5	B6	MIT	98	101	104	101	113	102	↓	<table><tr><td>27 days*</td><td>B1</td><td>B2</td><td>B3</td><td>B4</td><td>B5</td><td>B6</td></tr><tr><td>MIT</td><td>98</td><td>101</td><td>104</td><td>101</td><td>113</td><td>102</td></tr></table>						27 days*	B1	B2	B3	B4	B5	B6	MIT	98	101	104	101	113	102
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MIT	98	101	104	101	113	102																																			
<table><tr><td>150 days</td><td>B1</td><td>B2</td><td>B3</td><td>B4</td><td>B5</td><td>B6</td></tr><tr><td>NAT</td><td>< 5</td><td>< 5</td><td>160</td><td>< 5</td><td>< 5</td><td>< 5</td></tr></table>						150 days	B1	B2	B3	B4	B5	B6	NAT	< 5	< 5	160	< 5	< 5	< 5	<table><tr><td>150 days</td><td>B1</td><td>B2</td><td>B3</td><td>B4</td><td>B5</td><td>B6</td></tr><tr><td>NAT</td><td>< 5</td><td>< 5</td><td>20</td><td>< 5</td><td>< 5</td><td>< 5</td></tr></table>						150 days	B1	B2	B3	B4	B5	B6	NAT	< 5	< 5	20	< 5	< 5	< 5		
150 days	B1	B2	B3	B4	B5	B6																																			
NAT	< 5	< 5	160	< 5	< 5	< 5																																			
150 days	B1	B2	B3	B4	B5	B6																																			
NAT	< 5	< 5	20	< 5	< 5	< 5																																			
Cox.B ₃ virus i.p. 2.0 × 10 ⁸ (TCID ₅₀ /ml)							Cox.B ₁ virus i.p. 1.2 × 10 ⁸ (TCID ₅₀ /ml)																																		
↓	<table><tr><td>184 days</td><td>B1</td><td>B2</td><td>B3</td><td>B4</td><td>B5</td><td>B6</td></tr><tr><td>MIT</td><td>95</td><td>92</td><td>78</td><td>80</td><td>69</td><td>87</td></tr></table>						184 days	B1	B2	B3	B4	B5	B6	MIT	95	92	78	80	69	87	↓	<table><tr><td>184 days</td><td>B1</td><td>B2</td><td>B3</td><td>B4</td><td>B5</td><td>B6</td></tr><tr><td>MIT</td><td>87</td><td>84</td><td>59</td><td>89</td><td>88</td><td>94</td></tr></table>						184 days	B1	B2	B3	B4	B5	B6	MIT	87	84	59	89	88	94
	184 days	B1	B2	B3	B4	B5	B6																																		
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The pathological changes of myocardium: <u>SLIGHT</u>						The pathological changes of myocardium: <u>SEVERE</u>																																			

*Days after the inoculation

of NA each. Figure 1 shows the distribution of NAT against Cox.B group viruses. In normal controls and patients with heart diseases except myocarditis, there were 2 peaks at the titers of 20 and 80 folds. In patients with Cox.B viral myocarditis, NA against Cox.B viruses were divided into 2 groups according to changes of the titer in clinical courses: one was the changing titer group and the other was the unchanging titer group. Considering the combination of the changing group and the unchanging group of NAT, there were 3 peaks at the titer of 20, 80 and 1280 folds.

The Change of NAT and MIT, and Histopathological Findings in Guinea-Pigs Sensitized with Cox.B₃ Virus to Cox.B₃ (Homotype) or to Cox.B₁ (Heterotype) Virus

Eight guinea-pigs were served to the experiments. An equal volume of Cox.B₃ virus antigen solution was added to the Freund's complete adjuvant. Two ml of this mixture were intracutaneously injected into the footpad of these guinea-pigs. The value of MIT was within normal limits. One hundred and fifty days later, NAT

was measured. After these pretreatment, the guinea-pigs in A group were injected into peritoneum with Cox.B₃ virus (2.0×10^8 TCID₅₀/ml), and the guinea-pigs in B group were peritoneously injected with Cox.B₁ virus (1.2×10^8 TCID₅₀/ml). Five weeks after these procedures, MIT was measured.

Table III shows the typical results of 2 cases in these experiments. Macrophage migration was inhibited by adding either Cox.B₃ virus or Cox.B₅ virus in the guinea-pig A, and significantly inhibited by adding only Cox.B₃ virus in the guinea-pig B. The histopathological examination revealed perivascular inflammation and eosinophilic degeneration or necrosis. These pathological changes of myocardium in the guinea-pig B were more severe than those in the guinea-pig A.

The Results of NAT and MIT in 8 Patients with Cox.B Viral Myocarditis

NAT and MIT employing patient lymphocytes were examined in 8 patients with Cox.B viral myocarditis. The type of infected virus was estimated by the change of NAT. The results

TABLE IV CHANGES OF NT AND MIT IN 8 CASES OF COX.B VIRAL MYOCARDITIS

Case No. (Sex, Age)			CB1	CB2	CB3	CB4	CB5	CB6
1 (F) 64j	NT	23*	< 5	160	20	80	< 5	10
		37	< 5	160	20	80	< 5	40
	MIT	26	79	113	90	95	96	91 (%)
2 (M) 33j	NT	13	80	160	80	40	< 20	< 20
		21	160	40	160	< 20	< 20	< 20
	MIT	240	97	97	98	89	97	94
3 (M) 32j	NT	74	160	< 5	80	30	20	< 5
		86	640	< 5	120	40	20	< 5
	MIT	170	51	49	75	81	81	93
4 (M) 21j	NT	19	320	320	160	< 20	< 20	< 20
		39	160	30	160	80	20	< 5
	MIT	36	86	78	82	64	81	80
5 (M) 28j	NT	27	20	20	< 5	1280	< 5	10
		42	< 5	< 5	< 5	3840	< 5	15
	MIT	93	91	94	94	85	84	87
6 (F) 20j	NT	9	< 5	< 5	80	< 5	< 5	< 5
		23	< 5	< 5	20	< 5	< 5	< 5
	MIT	32	80	104	70	116	118	110
7 (M) 49j	NT	18	10	30	320	60	40	< 5
		48	10	20	80	20	20	< 5
		94	< 5	20	20	20	20	< 5
	MIT	96	75	68	78	65	67	78
8 (F) 30j	NT	24	< 5	< 5	< 5	80	< 5	< 5
		42	< 5	< 5	< 5	80	< 5	< 5
	MIT	52	87	80	86	87	83	87

*Days after the onset of symptom

were shown in Table IV.

Cell-mediated immunity (CMI) estimated by MIT was enhanced not only in the same type but also in the other types of Cox.B viruses. For example, Case 3 showed a four-fold rise of NAT against Cox.B₁ virus and was diagnosed as Cox.B₁ viral myocarditis. CMI was augmented in

Cox.B₂ and B₃ as well as Cox.B₁. Almost same findings were observed in Cases 4 and 6.

DISCUSSION

As to the occurrence of viral myocarditis, it is suggested that the mechanism in the adult differs from that in the newborn.⁵ Namely, adult

myocarditis may be produced by allergic or autoimmune reactions against the initial infection. Viremic stage has been ended and antibody is proved in the serum despite low levels. In case of such situations, the isolation of virus is much difficult. On the other hand, newborn myocarditis may be provoked by the direct invasion of the virus into myocytes, and original virus could be isolated at a high rate.¹⁰

In our data, a few cases of Cox.B viral myocarditis demonstrated the increase of the antibody titer against only one type of Cox.B group viruses. Many cases, however, had the antibody against the other types of Cox.B group viruses which did not show the change of the antibody titer by NAT. These findings support the existence of previous infection by the other types of Cox.B group viruses. On the contrary, in all cases of Cox.B viral meningitis, the possession and rising of antibody are proved against only one type of Cox.B group viruses.¹¹ Thus, it is essential to the occurrence of adult myocarditis that patients are infected by one of Cox.B viruses and immunized against not only the same type but also the other types of Cox.B group viruses.

It has been pointed out that CMI reactions as well as humoral immunity represented by neutralization play an important role in the pathogenesis of the heart disease induced by viral infection.^{12,13} However, experimental evidences have scarcely been obtained.

MIT, one of the few available satisfactory methods for the *in vitro* study of delayed hypersensitivity,¹⁴ is the most quantitative, specific and efficient method to examine the movement of CMI. According to the report of Tanaka,¹¹ the existence of mutual antigenicity would participate in CMI in 6 types of Cox.B viruses. The type peculiarity which is seen in humoral antibody may not be distinct in CMI. In this study, the type of infectious virus which was estimated by NAT had highly type specificity. In many cases, CMI using MIT was enhanced not only against the same type but also against the other types of Cox.B group viruses.

Accordingly, our data would immunologically support the hypothesis of Cayler et al⁵ concerning the occurrence of myocarditis: namely, newborn myocarditis may be evoked by the direct invasion of the Cox.B viruses into myocytes. However, many cases are inapparent infections,

and aseptic meningitis is occurred in a few cases. Both NAT and CMI are enhanced in the same type of infectious virus. On the other hand, CMI in some cases is augmented in the other types of Cox.B group viruses. When they are reinfected with the same type or the other types of Cox.B group viruses, myocarditis would occur through the participation of CMI. On the other hand, adult myocarditis would be occurred by autoimmune reactions which are stimulated by viral infections. Namely, CMI as well as humoral immunity may play an important role in the occurrence of adult myocarditis.

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