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ABSENCE OF A CORRELATION BETWEEN HUMORAL AND CELLULAR RESPONSES TO VACCINIA VIRUS AND PRODUCTS OF THE MAJOR HISTOCOMPATIBILITY COMPLEX IN RHESUS MONKEYS

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Sixty-two Rhesus monkeys were tested at different times after vaccinia virus infection for virus-specific induction of lymphocyte proliferation in vitro or antibody production in vivo. No association was found between identifiable RhLA-controlled antigens and the strength of the cellular proliferative and/of humoral response.

MHC disease association vaccinia virus RhLA

INTRODUCTION

It is generally accepted [13,15] that antibody responses are regulated by immune response (Ir) genes, some of which have been shown to be part of the major histocompatibility complex (MHC) [14]. A relationship has also been found between the MHC and various diseases. For virus infections, this relationship between the MHC and the cellular immune response involved mainly an 'MHC restriction' or selective lysis of infected target cells by the specific cytotoxic effector cells which share MHC products with them.

Evidence has been found for an association, in man, between certain HLA antigens and the immune response to vaccinia virus antigens [6], influenza virus type A [5], rubella virus [11], tetanus [18] and streptococcal antigens[9]. It seemed interesting therefore to investigate whether a similar association could be found in another outbred

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Abbreviations used: MHC, major histocompatibility complex; HLA, human leucocyte antigen; RhLA-A, B, A or B locus of the MHC of the Rhesus monkey; DR, D-related locus of the MHC (DR antigens are closely related with the D antigen which have an overriding influence in mixed lymphocyte cultures).

species. In the study reported here, Rhesus monkeys were vaccinated and tested for their immune response as previously described [6]. Vaccinia virus infections are mild and have been used in several studies investigating cellular immune responses. Thus, vaccinia virus has been shown to elicit well-defined MHC-restricted cytotoxic T cell responses in mice [3,7] but not in man [17]. Since the MHC of the Rhesus monkey (RhLA) has been thoroughly studied [2], experiments were performed to determine whether there is an association between certain MHC-controlled antigens and the level of in vivo antibody responses or in vitro virus-specific proliferative reactivity against vaccinia infection in this primate species.

EXPERIMENTAL

Imported and presumably unrelated adult male and female Rhesus monkeys were used for the experiments. Sixty-two animals found to be negative when tested against vaccinia virus antigen in a lymphocyte transformation test (LTT) and also for antibodies against vaccinia virus were selected. For the vaccination of Rhesus monkeys, one stock of vaccinia virus strain WR was used. The virus was routinely propagated and titrated on mouse L929 cells. The particular stock used for the vaccination contained 2.0×10^7 plaque-forming units/ml (p.f.u.$\cdot$ml^{-1}). After careful shaving of the chest, the animals were inoculated intracutaneously at multiple sites. The total volume of virus suspension inoculated per animal was 1.0 ml. Pre- and post-infection sera were collected from all animals for the determination of antivaccinia virus antibodies. The animals received no other treatment during the experiment. They were kept in quarantine and the development of vaccinia virus-specific skin lesions was scored daily by two investigators working independently. After 14 days, all skin lesions had resolved, leaving only small scars, and the monkeys were returned to the colony.

A modified version of Kissmeyers' one-stage microcytotoxicity test was employed to determine the antigens of the RhLA-A and B loci [1,12]. Briefly, lymphocytes were isolated by a Ficoll–Isopaque gradient technique and incubated for 15 min at 37°C with typing sera and complement. The supernatant was then removed, trypan blue solution added to the wells, and the plates again incubated for 30 min at room temperature. Finally, buffered formalin was added and the reaction was checked by the use of an inverted microscope with bright field illumination. The methods used for producing, screening and selecting the antigens have been described previously [20]. To determine D-related antigens, B cell-enriched lymphocyte suspensions were used [19] in a routine two-stage microcytotoxicity test [16].

For lymphocyte transformation tests (LTT), samples of sterile heparinized blood were taken from each animal before, 5 weeks after, and again between 9 and 16 weeks after infection. The lymphocytes were collected by centrifugation on a Ficoll–Hypaque gradient [4]. A freeze-dried suspension of rabbit kidney cells infected with vaccinia virus was used as antigen [10]. The virus was heat-inactivated at 56°C for 30 min before being added to the test wells. Fifty μl of virus-containing suspension were then added to the

wells of microtiter plates containing 3×10^5 lymphocytes in 200 μl RPMI 1640 medium with 25 mM Hepes buffer, supplemented with 20% heat-inactivated (56°C for 30 min) serum of a pool of three donor monkeys and 100 $\mu g \cdot ml^{-1}$ penicillin and streptomycin. After 5 days of culture at 37°C in a moist atmosphere with 5% CO_2, 0.5 μCi of [2-^{3}H]-thymidine was added to the culture and 24 h later the [2-^{3}H] thymidine uptake was measured in a liquid scintillation counter. As a control, 3×10^5 lymphocytes were cultured in the above described medium with the addition of 50 μl of a lysate of rabbit kidney cells not infected with vaccinia virus but otherwise treated as described above. The results of these test were expressed as the stimulation index, calculated by the formula:

$$\frac{[^3\text{H}]\text{ thymidine incorporation in test sample}}{[^3\text{H}]\text{ thymidine incorporation in control sample}}$$

The titration of antivaccinia virus antibodies was kindly performed by Dr. A.C. Hekker (RIV, Bilthoven, The Netherlands), by the use of the immunofluorescence technique as described elsewhere [8]. Before infection all animals showed titers of $\leqslant 1 : 8$.

In an initial experiment, we investigated whether Rhesus monkeys previously vaccinated with vaccinia virus would respond to the same antigen in vitro in the LTT. To select an optimal virus dose, three groups of three Rhesus monkeys each were infected with virus doses of 4×10^9, 4×10^7 and 4×10^6 p.f.u., respectively. Five weeks after vaccination, lymphocytes were stimulated in vitro using heat-inactivated virus at different doses. The results of this pilot test showed that the best discrimination of the data was obtained at a dose of 4×10^7 p.f.u.$\cdot ml^{-1}$ vaccinia virus for in vivo inoculation and 10^5 p.f.u.$\cdot ml^{-1}$ for in vitro stimulation of lymphocytes.

Table 1 shows the results of the first series of experiments in which 38 unrelated Rhesus monkeys were tested for their in vitro immune response after infection with vaccinia virus strain WR. On measuring the humoral immune responses, all animals exhibited seroconversion with antibody titers of 1/256 to 1/512 at 3 weeks after infection. One animal (No. 29) showed a lower titer of 1/64. A larger variation was observed in the cellular in vitro immune response (Table 1). Monkeys responding at all times in the LTT with a stimulation index below 5.0 were regarded as low responders. Of 38 animals tested, 12 gave clearly positive responses, four moderately positive ones and 22 showed no response. Two animals (Nos. 19 and 21) of the 12 clearly positive responders were excluded from the evaluation of the results because of their in vitro immune responsiveness at week 0. Similar proportions of early and late responders in both groups of low and high responders were observed. With the exception of two animals (Nos. 19 and 20) which became negative after an initial positive reaction, all early responders remained positive. No sex relationship for animals responding or not responding in vitro was found. Additionally, there were no significant differences in the skin reactions to vaccinia virus infection within and between the groups of in vitro high

TABLE 1

In vitro stimulation of lymphocytes and humoral antibody responses in vaccinia virus-infected Rhesus monkeys

Monkey number	Sex[a]	Lymphocyte stimulation index at weeks			Antibody titres at week 3[b]	RhLA antigens				DR antigens	
		0	5	9–16		B locus		A locus			
High responders											
13	f	1.0	4.8	10.4	256	5	10	*11*	–	7	–
28	f	1.0	1.0	10.4	256	1	28	*11*	14	5	–
15	m	1.0	1.3	9.8	512	6	19	*11*	25	3	5
18	m	1.0	8.4	8.9	256	3	6	*11*	25	2	8
17	m	1.0	6.1	5.4	512	9	–	*11*	13	3	–
9	m	1.0	2.1	5.2	256	9	–	*11*	–	2	–
14	f	1.0	ND[c]	13.5	512	9	28	17	32	5	–
8	m	1.0	10.9	7.0	256	1	3	18	24	2	5
7	m	1.0	2.2	10.3	256	6	19	18	26	6	8
31	f	1.0	ND	9.2	256	5	6	25	26	2	6
1	f	1.0	3.4	8.1	512	6	–	13	32	1	8
6	f	1.0	8.6	ND	512	1	19	26	–	2	6
27	m	1.0	1.1	6.8	512	1	28	26	32	5	–
20	f	1.0	5.4	2.6	512	10	23	18	26	3	–
19	f	4.6	13.6	1.0	256	6	10	13	26	4	–
21	f	4.6	5.9	9.4	512	6	19	13	13	4	–
Low responders											
11	f	2.2	3.8	1.9	256	10	28	13	*29*	1	8
22	m	1.0	2.7	4.9	512	6	–	17	*29*	4	–
25	m	1.0	3.3	1.0	256	9	–	17	*29*	–	–
26	f	2.4	2.1	1.0	256	6	26	26	*29*	3	–
38	f	1.0	1.0	1.0	512	6	33	17	*29*	3	–
3	m	1.0	1.0	3.9	256	6	–	*11*	*29*	2	3
2	f	1.0	1.0	4.6	512	9	10	13	34	1	6
4	f	2.5	3.5	ND	512	6	9	24	32	2	4
5	m	1.0	ND	1.9	512	9	19	24	–	3	6
10	f	1.0	3.9	4.5	256	6	27	13	34	3	–
12	f	1.0	2.2	3.2	512	6	–	13	25	3	6
16	f	1.0	2.2	ND	512	9	10	2	26	2	–
23	m	1.0	2.5	ND	512	5	23	18	–	2	6
24	m	1.0	1.1	1.7	256	3	5	*11*	18	2	3
29	m	1.0	1.1	4.6	64	19	28	17	24	2	3
30	m	1.0	2.5	1.0	512	19	28	34	–	3	–
32	f	1.0	ND	2.5	256	28	–	*29*	34	7	8
33	f	1.0	ND	1.0	512	–	–	17	26	3	–
35	f	1.0	1.7	1.0	512	19	28	1[illegible]	18	1	5
36	m	1.0	1.0	1.7	512	6	–	13	26	1	6
37	m	1.0	1.0	1.0	512	33	–	18	20	5	–
39	m	1.0	2.2	4.3	256	6	–	26	31	1	–

[a] m = male; f = female. [b] Reciprocal of end-point dilution. [c] Not determined.

and low responders. With regard to the RhLA-SD phenotypes in the group of high responders, six animals carried antigen 11 of the A locus, while only two out of the 22 low responders expressed this particular antigen. Antigen 29 of the A locus occurred only in animals that were negative in their in vitro response to vaccinia virus antigen. Statistical analysis (2 X 2 chi square test with Yates' correction for small numbers) revealed that both prevalences were not significant if the number of A and B locus antigens were taken into consideration. Since this did not eliminate the possibility of a weak correlation between RhLA antigens and reactivity to vaccinia virus, a second experiment was done which was designed in a prospective fashion. Eleven animals carrying antigen A 11 but not A 29, 11 animals with antigen A 29 but not A 11, and two animals carrying both of these A locus antigens were tested for in vitro responsiveness after primary vaccination.

Table 2 shows the results, which also failed to reveal an association of antigen A 11 with the high responder status and antigen A 29 with the low responder status ($P > 0.6$ according to a 2 X 2 chi square test). Most animals showing a positive reaction 9 weeks after infection remained positive; exceptions were monkeys Nos. 15 and 19, which showed a decrease in the stimulation index to below 5.0. Four animals negative in their response after 5 and 9 weeks became positive by week 16. The humoral immune response (which is not shown in the table) showed the same pattern as observed in the first experiment, namely, antibody titers higher than or equal to 1/256. Again, no correlation of responsiveness with sex was found in this experiment. Also no association of DR antigens of RhLA with in vitro reactivity was found.

In summary, employing a lymphocyte transformation test we showed that lymphocytes of vaccinia virus-infected Rhesus monkeys responded to the vaccinia antigen in vitro, while those of non-vaccinated monkeys did not. In the group of high responders the prevalences of early and late responses were similar to those in the group of low responders. An initial indication for a possible association between RhLA antigen A 11 and responder status and between antigen A 29 and non-responder status could not be confirmed.

In man, evidence in favour of an association between a particular MHC product and the strength of a cellular immune response during vaccinia virus vaccination has been given by De Vries et al. [6]. In particular, evidence was found for an association between a negative in vitro response to the viral antigen and the HLA antigen Cw3. There are no satisfactory explanations for the apparent discrepancy between the results obtained in man and in the Rhesus monkeys. One explanation, although not very likely, could be the use of different strains of vaccinia virus for the vaccination in the two species. Another explanation might be that no equivalent for the human C locus has been identified in Rhesus monkeys until now. Finally, the selection of test individuals for the human study might explain the differing results. There may have been reasons why the test individuals in De Vries' report had not been vaccinated at a young age, e.g. they may have been excluded from vaccination against smallpox because they suffered from allergic disease at the time or had a temporarily impaired immune reactivity. Therefore, the group might be regarded as selected. In contrast, none of the animals in the current study were

TABLE 2

In vitro cellular immune response to vaccinia virus in animals preselected for the presence of RhLA antigens A 11 and A 29

Monkey number	Lymphocyte stimulation index at weeks				RhLA antigens				DR antigens	
	0	5	9	16	B locus		A locus			
Monkeys positive for A 11, negative for A 29										
109	3.0	12.6	6.9	14.0	9	19	*11*	24	5	–
106	1.0	2.0	10.2	7.6	9	10	*11*	13	2	3
108	1.0	1.0	5.8	9.1	9	10	*11*	13	2	3
111	1.0	2.2	8.6	ND	6	9	*11*	26	4	–
101	1.0	1.2	3.2	8.0	9	10	*11*	13	2	3
110	ND	1.0	4.4	7.2	1	10	*11*	–	1	3
107	1.0	1.3	4.4	6.6	1	9	*11*	–	1	2
104	1.0	1.0	2.8	5.6	19	33	*11*	17	2	–
102	1.0	1.1	1.0	1.0	3	6	*11*	23	3	–
103	1.0	1.4	3.1	4.6	6	6	*11*	14	3	–
105	1.0	1.1	ND	2.8	1	9	*11*	26	5	2
Monkeys negative for A 11, positive for A 29										
112	1.0	4.8	25.1	31.1	19	19	26	*29*	5	–
117	1.0	3.9	20.1	15.4	9	22	24	*29*	1	3
114	1.0	3.3	19.1	1.0	6	9	17	*29*	1	2
120	1.0	1.0	16.6	17.1	10	19	26	*29*	1	8
116	1.0	7.1	14.7	13.5	10	19	32	*29*	5	–
119	2.8	1.3	12.8	3.5	6	24	–	*29*	3	8
113	1.0	3.9	2.0	7.4	6	–	14	*29*	8	–
115	1.0	1.2	6.3	1.0	9	13	19	*29*	1	3
121	2.7	6.3	4.1	5.9	10	10	24	*29*	1	4
118	1.0	1.0	3.5	3.8	9	10	24	*29*	1	3
122	3.8	1.2	2.6	3.8	6	–	14	*29*	8	–
Monkeys positive for both A 11 and A 29										
123	1.0	10.4	6.5	4.9	19	–	*11*	*29*	4	8
124	1.0	2.2	7.7	5.1	6	10	*11*	*29*	2	8

excluded, with the exception of those in which antivaccinia virus antibodies were already present before the experimental vaccination.

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