Kinetics of the Immune Response of Rabbits to Lower Trypanosomatidae Antigens

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(Accepted for publication 1 July 1967)

SUMMARY

Experiments tracing the kinetics of the hyperimmune response in rabbits injected with several lower trypanosomatids (*Crithidia fasciculata* (Anopheles strain), *C. fasciculata* (Culex strain, Wallace isolate), *C. fasciculata* (Culex strain, Nöller isolate), *Crithidia* sp. from *Euryophthalmus davisii*, *Blastocrithidia leptocoridis*, *Leptomonas* sp. from *Dysdercus* and *L. mirabilis*) mixed with Freund's complete adjuvant revealed a slow increase in homologous agglutinating titre, followed by a lasting plateau titre. Cross-reactions tested at various homologous titres showed that cross-reacting specificity increased as the titre increased. At peak agglutinating titre, sera were at their maximum specificity and remained like that during the lasting plateau period. Precipitins appeared before agglutinins and faded as the homologous agglutinating titres increased. Precipitins, in contrast to agglutinins, were specific even when the agglutinating titres were low and non-specific. By analogy with work done by other investigators on identification of serum fractions associated with non-protozoan antigens in which immune kinetics, globulin generation, or globulin specificity were traced, we suggest that the early-appearing non-specific agglutinins may be composed of 19S globulins and the late-appearing specific agglutinins and specific precipitins may be composed of 7S globulins.

INTRODUCTION

There have been only four reports in which relationships among the lower Trypanosomatidae have been traced by their immunological affinities (Clark, 1958; McGhee & Hanson, 1963; Noguchi, 1926; Vitetta & Guttman, 1967). Differences in the results of various investigators have led to conflicting statements about group relationships. Some examples are: (1*a*) within the genus *Crithidia*, all organisms isolated from hemipterons are more closely related immunologically to each other than are all organisms originally isolated from dipterons (McGhee & Hanson, 1963), or (1*b*) immunological relationships are independent of host-type (Vitetta & Guttman, 1967); (2*a*) Culex and Anopheles strains of *Crithidia fasciculata* are identical (Noguchi, 1926), (2*b*) Anopheles and only one but not another Culex strain of *C. fasciculata* are identical (Clark, 1958), (2*c*) from immunological and biochemical evidence, the Anopheles and both Culex strains of *C. fasciculata* are distinct (Vitetta & Guttman, 1967).

In only one of the aforementioned reports (Vitetta & Guttman, 1967) was there used the combination of (a) an antibody-generating system said to produce the most physico-chemically homogeneous antibodies (Crowle, 1963; Kabat & Mayer, 1961);

(b) high-titre antisera (equal to or greater than 1/2560) for testing all cross-sections; (c) routine inactivation of complement. Since Vitetta & Guttman (1967) prepared antibodies by a method which included the use of Freund's complete adjuvant (a mixture which not only enhances production of high-titre antiserum but also lengthens the time required before peak titre is reached), we report here on the kinetic tracking of antibody production during the course of the hyperimmune response in rabbits to antigens mixed the Freund's adjuvant and on cross-reactivity of sera before, at, and after peak homologous titre.

METHODS

The organisms used as antigens in these studies were *Crithidia fasciculata* (Anopheles strain) ATCC 11745; *C. fasciculata* (Culex strain, Wallace isolate) ATCC 12857; *C. fasciculata* (Culex strain, Nöller isolate) ATCC 12858; *C. luciliae* ATCC 14765; *C. oncopelti* (with endosymbiote) ATCC 12982; *Crithidia* sp. from *Euryophthalmus davisi*, ATCC 14766; *Blastocrithidia leptocoridis*; *Leptomonas* sp. from *Dysdercus suturellus*, and *L. mirabilis*.

Methods already reported (Vitetta & Guttman, 1967) were used for preparation of antigens, mixture of antigen with Freund's complete adjuvant for hyper-immunization, generation of antibodies in rabbits and procedures for agglutination tests.

Precipitin tests. All precipitin tests were done in Ouchterlony double diffusion plates (Hyland Laboratories, Los Angeles, California). Disrupted-cell antigen suspensions were placed in the central well and undiluted sera placed in the peripheral wells. The plates were covered with their own plastic lids to prevent drying and were incubated at room temperature (25°) for 24 hr. They were then washed in cold 0.85% (w/v) NaCl solution for 24–48 hr to remove excess antigen and serum and then scanned at $\times 100$ magnification for precipitin lines. The numbers and intensity of precipitin lines were recorded for each antiserum challenged with homologous antigen. Cross-reactions were determined by using sera which showed the most intense and/or greatest number of lines with homologous antigen.

RESULTS

The usual pattern of agglutinating hyperimmune response in rabbits following injection of antigen mixed in Freund's complete adjuvant was an increase in titre beginning 1–20 days after the last antigen injection and continuing to rise for 10–40 more days (Fig. 1). After peak titre was reached, there was a slight decrease in titre and persistence of the agglutinating response at this plateau for at least 140 days (experiments were terminated at that time). Such a pattern of slow rise of titre and maintenance of high titre is typical for adjuvant-injected animals (Uchitel & Khasman, 1965). The one exception to formation of a stable agglutinating titre in our experiments was with one *Crithidia* sp. from *Euryophthalmus* antiserum which reached a rather low peak titre and then decreased rapidly to zero (Figure 2).

For all antisera tested, there was a distinct pattern of relationship between precipitins and agglutinins which was unrelated to either the particular antigen used or to the type of cell preparation (i.e. whole or disrupted cells). Precipitins appeared before agglutinins and usually were most apparent when agglutinating titres were still low (Table 1).

Although all precipitating responses declined as the agglutinating titres increased (Table 1), the precise timing was not constant: precipitins disappeared (a) before peak agglutinating titre in two antisera (anti-*C. fasciculata* (Culex strain) serum and anti-*Leptomonas* sp. from *Dysdercus suturellus* serum), (b) slightly after peak agglutinating titre in three antisera (two *C. fasciculata* (Culex strain) sera and anti-*B. leptocoridis* serum), and (c) persisted in two other antisera (anti-*Leptomonas* sp. from *Dysdercus suturellus* serum and anti-*L. mirabilis* serum).

Fig. 1

Fig. 1. Kinetics of antibody generation to whole *Crithidia fasciculata* (Culex strain, Wallace isolate). Similar kinetics were observed in 15 other antisera generated against lower trypanosomatid antigens.

Fig. 2

Fig. 2. Kinetics of antibody generation to disrupted organisms of a *Crithidia* sp. (from *Euryophthalmus davisi*).

In our previous study (Vitetta & Guttman, 1967) all agglutinating cross-reactions were determined at peak or plateau homologous titre. We now show (Table 2) the striking decrease in agglutinating cross-reactions concomitant with increase in homologous titre. For antisera to whole or disrupted *C. fasciculata* (Culex strain, Wallace isolate), whole *Leptomonas* sp. from *Dysdercus suturellus*, or whole *B. leptocoridis*, four series in which cross-reactions were followed during the whole sequence of rising, plateau and decreasing titre (Table 2), antibody specificity increased as the homologous titre increased. This specificity was maintained and strengthened at peak and post-peak homologous titres. Although there were insufficient data to follow the development of cross-reacting antibody specificity for other organisms, some generalizations can be made:

(1) At very low agglutinating titres, agglutinating cross-reactions were high and non-specific (Table 3a).

(2) Cross-reactivity declined as the homologous agglutinating titre neared a peak, i.e. antibody specificity increased with titre (Table 2).

(3) At peak titre, plateau titre, and decreasing titre after peak, agglutinating cross-reactions remained constantly specific, despite the changes in homologous titre (Table 3*b*).

In contrast to lack of specificity of agglutinating antibodies, precipitating cross-

Table 1. *Kinetics of the production of agglutinating and precipitating antibodies*

Antiserum to	Rabbit no.	Days since last injection	Agglutinating titre	Precipitating lines (no. and intensity)
<i>C. fasciculata</i> (Culex, Wallace)	1	12	1/40	1 faint
		28	1/1280	1 faint
		40	1/5120-1/10240	1 faint
		140	1/5120	0
	2	15	0	0
		20	0	1 faint
		30	1/1280	1 faint
		32	1/5120	1 faint
		40	1/1280	1 faint
		50	1/1280	0
	3	8	1/1	1 sharp
		14	1/10	1 faint
		21	1/80	1 faint
		27	1/640	1 faint
		36	1/2560	0
<i>Leptomonas</i> from <i>Dysdercus</i> suturellus	1	10	0	2 sharp
		14	0	2 faint
		21	1/80-1/160	1 sharp
		27	1/320	1 sharp
		30	1/1280	1 sharp
		37	1/2560-1/5120	1 faint
		40	1/5120	1 faint
	2	10	0	2 sharp
		14	0	1 sharp
		21	0	1 faint
<i>B. leptocoridis</i>		30	1/320	0
		5	0	2 very sharp
		10	0	2 sharp
		15	1/640-1/1280	2 faint
		25	1/640-1/1280	1 faint
		30	1/2560	1 faint
		48	1/5120	1 faint
		55	1/10240	1 faint
		110	1/2560	1 faint
		120	1/2560	1 faint
		140	1/1280	0
<i>L. mirabilis</i>		5	0	2 sharp
		10	0	2 sharp
		20	1/640-1/1280	2 faint
		30	1/1280	2 faint
		40	1/10240	1 faint
		56	1/10240	1 faint
		63	1/5120	1 faint
		69	1/2560	1 faint

reacting antibodies are very specific when the homologous agglutinating titre is low and remain specific during the course of the precipitating immune response (Table 4; see also Vitetta & Guttman, 1967).

Table 2. *A comparison of percentage agglutinating cross-reaction at indicated homologous agglutinating titres*

Antiserum to	Whole antigen	Homologous agglutination titre at which cross-reactions were done.						Post- peak titre
		Rising titres						
		1/20	1/640	1/1280	1/2560	1/5120	1/10240	
Whole <i>C. fasciculata</i> (Culex strain, Wallace isolate)	<i>C. from Euryophthalmus davisi</i>	100	100	.	.	25*	.	25
	<i>L. from Dysdercus suturellus</i>	100	50	.	.	25*	.	50
	<i>L. mirabilis</i>	100	100	.	.	100*	.	50
	<i>B. leptocoridis</i>	100	100	.	.	100*	.	25
Disrupted <i>C. fasciculata</i> (Culex strain, Wallace isolate)	<i>C. from Euryophthalmus davisi</i>	.	.	100	.	12	12*	.
	<i>L. from Dysdercus suturellus</i>	.	.	100	.	25	25*	.
	<i>L. mirabilis</i>	.	.	100	.	50	50*	.
	<i>B. leptocoridis</i>	.	.	50	.	12	12*	.
Whole <i>Leptomonas</i> from <i>Dysdercus</i> <i>suturellus</i>	<i>C. from Euryophthalmus davisi</i>	.	100	50	.	12*	.	.
	<i>C. fasciculata</i> (Culex strain, Wallace isolate)	.	100	100	.	25*	.	.
	<i>L. mirabilis</i>	.	100	100	.	25*	.	.
	<i>B. leptocoridis</i>	.	100	100	.	50*	.	.
Whole <i>Blastocrithidia</i> <i>leptocoridis</i>	<i>Crithidia</i> sp. from <i>Euryophthalmus davisi</i>			100	100	25	12*	.
	<i>C. fasciculata</i> (Culex strain, Wallace isolate)			100	100	50	25*	.
	<i>Leptomonas</i> sp. from <i>Dysdercus suturellus</i>			100	50	50	50*	.
	<i>L. mirabilis</i>			100	50	50	50*	.

* Peak homologous agglutinating titre.

Table 3a. *Percentage agglutinating cross-reaction at low (pre-peak) homologous agglutinating titres*

Antiserum to	Peak titre	Titre when cross-reactions tested	Antigen	% cross-reaction
Whole <i>C. fasciculata</i> (Culex strain, Nöller isolate)	1/5120-1/10240	1/1280	<i>C. fasciculata</i> (Culex strain, Wallace isolate)	100
			<i>C. fasciculata</i> (Anopheles strain)	100
			<i>C. from Euryophthalmus davisi</i>	100
			<i>C. luciliae</i>	100
			<i>C. oncopelti</i> (endosymbiote infected)	100
			<i>L. from Dysdercus suturellus</i>	25
Whole <i>Crithidia</i> from <i>Euryophthalmus</i>	1/2560	1/640	<i>C. fasciculata</i> (Anopheles strain)	100
			<i>C. fasciculata</i> (Culex strain, Wallace isolate)	100
			<i>L. from Dysdercus suturellus</i>	100
			<i>C. fasciculata</i> (Culex strain, Nöller isolate)	100
			<i>C. luciliae</i>	100
			<i>C. oncopelti</i> (endosymbiote infected)	100
Whole <i>C. luciliae</i>	1/5120-1/10240	1/1280	<i>C. fasciculata</i> (Anopheles strain)	100
			<i>C. fasciculata</i> (Culex strain, Wallace isolate)	100
			<i>C. from Euryophthalmus davisi</i>	50
			<i>C. fasciculata</i> (Culex strain, Nöller isolate)	50
			<i>L. from Dysdercus suturellus</i>	100
			<i>C. oncopelti</i> (endosymbiote infected)	50

Table 3*b*. Percent agglutinating cross-reaction at peak and post-peak homologous agglutinating titres

Antiserum to	Peak titre	Titre when cross-reactions tested	Antigen	% cross-reaction
Whole <i>C. fasciculata</i> (Culex strain, Wallace isolate)	1/10240	1/10240	<i>C. fasciculata</i> (Anopheles strain)	50
			<i>C. from Euryophthalmus davisii</i>	6
			<i>C. fasciculata</i> (Culex strain, Nöller isolate)	50
			<i>L. from Dysdercus suturellus</i>	6
			<i>C. oncopelti</i> (endosymbiote infected)	50
			<i>C. luciliae</i>	6
Disrupted <i>C. fasciculata</i> (Culex strain, Nöller isolate)	1/5120	1/5120	<i>C. fasciculata</i> (Culex strain, Wallace isolate)	50
			<i>C. fasciculata</i> (Anopheles strain)	100
			<i>C. from Euryophthalmus davisii</i>	25
			<i>C. luciliae</i>	25
			<i>C. oncopelti</i> (endosymbiote infected)	25
			<i>L. from Dysdercus suturellus</i>	25
Whole <i>C. fasciculata</i> (Anopheles strain)	1/5120	1/5120	<i>C. fasciculata</i> (Culex strain, Wallace isolate)	100
			<i>C. from Euryophthalmus davisii</i>	25
			<i>L. from Dysdercus suturellus</i>	6
			<i>C. fasciculata</i> (Culex strain, Nöller isolate)	50
			<i>C. luciliae</i>	12
			<i>C. oncopelti</i> (endosymbiote infected)	50
Disrupted <i>C. fasciculata</i> (Culex strain, Wallace isolate)	1/5120	1/5120	<i>C. fasciculata</i> (Anopheles strain)	100
			<i>C. from Euryophthalmus davisii</i>	12
			<i>C. fasciculata</i> (Culex strain, Nöller isolate)	50
			<i>C. luciliae</i>	25
			<i>L. from Dysdercus suturellus</i>	25
			<i>C. oncopelti</i> (endosymbiote infected)	50
Disrupted <i>C. from Euryophthalmus davisii</i>	1/2560	1/320	<i>L. from Dysdercus suturellus</i>	12
			<i>L. mirabilis</i>	25
			<i>B. leptocoridis</i>	12
			<i>C. fasciculata</i> (Culex strain, Wallace isolate)	25
Disrupted <i>L. from Dysdercus suturellus</i>	1/2560	1/640	<i>C. fasciculata</i> (Anopheles strain)	50
			<i>C. fasciculata</i> (Culex strain, Wallace isolate)	25
			<i>C. from Euryophthalmus davisii</i>	100
			<i>C. fasciculata</i> (Culex strain, Nöller isolate)	25
			<i>C. luciliae</i>	25
			<i>C. oncopelti</i> (endosymbiote infected)	25
Disrupted <i>L. mirabilis</i>	1/5120–1/10240	1/5120	<i>C. fasciculata</i> (Culex strain, Wallace isolate)	50
			<i>L. from Dysdercus suturellus</i>	25
			<i>B. leptocoridis</i>	100
			<i>C. from Euryophthalmus davisii</i>	25

DISCUSSION

Although it is clear that the specificity of precipitating antibodies was acquired early in the course of the immune response while the specificity of agglutinating antibodies was still changing in the direction of increased specificity during the same period (Tables 2, 3), the biochemical events leading to the changes in specificity are not yet clear. Two possible explanations are: (i) there is an increasing modification of a particular class of antibody molecules as the titre rises; (ii) there is independent synthesis of different classes of antibody proteins each of which differs with respect to specificity and probably to gross molecular structure.

It has now been established by numerous workers that at least two types of globulins are generated after primary inoculation with viral, bacterial, or foreign antigen. The first antibodies generated are 19S globulins (gamma M or macroglobulins) which are later replaced by lighter 7S globulins (gamma G or gamma globulins) (Bauer & Stavitsky, 1961; Daniel, 1965; Benedict, Brown & Ayengar, 1962; Nisonoff & Thorbecke, 1964; Svehag & Mandel, 1964). Normal and immune 7S globulins contain

Table 4. *Precipitating cross-reactions between disrupted antigen and antisera against disrupted cells*

The experiments were done with sera which showed the most intense and/or greatest number of lines with homologous antigen.

Antiserum to	Antigens				
	<i>Crithidia</i> sp. <i>fasciculata</i> (Culex-Wallace)	from <i>Eury-</i> <i>ophthalmus</i> <i>davisi</i>	<i>Leptomonas</i> sp. from <i>Dysdercus</i> <i>suturellus</i>	<i>mirabilis</i>	<i>Blasto-</i> <i>crithidia</i> <i>Leptocoridis</i>
<i>Crithidia fasciculata</i> (Culex strain, Wallace isolate)	+	—	—	—	—
<i>Crithidia</i> sp. from <i>Euryophthalmus davisi</i>	—	+	—	—	—
<i>Leptomonas</i> sp. from <i>Dysdercus suturellus</i>	—	—	+	—	—
<i>L. mirabilis</i>	—	—	—	+	—
<i>Blastocrithidia leptocoridis</i>	—	—	—	—	+

two identical light (L) chains linked by disulphide bonds to two identical heavy (H) chains (Edelman & Benacerraf, 1962; Edelman, Benacerraf & Ovary, 1963; Edelman & Gally, 1964; Nossal, Svenberg, Ada & Austin, 1964; Fougereau & Edelman, 1965). There are several alternative classes of H chains which can combine with L chains to make the whole 7S molecule (Potter, Apella & Geisser, 1965). Antibody specificity is dependent upon the amino acid sequence at or near the two H-L interaction sites (Almeida, Cinader & Howatson, 1963; Buckley, Whitney & Tanford, 1963; Koshland & Engelberger, 1963; Roholt, Onoue & Pressman, 1964; Metzger, Wofsy & Singer, 1964; Roholt, Radzimski & Pressman, 1963; Titani & Putnam, 1965; Whitney & Tanford, 1965). The macroglobulin has a molecular weight of one million and contains the same L chains as does the globulin, but different H chains (Edelman & Gally, 1964; Nossal, Svenberg, Ada & Austin, 1964). Since the gamma G globulin and gamma M macroglobulin have different primary structures, the macroglobulins are not merely polymers of smaller 7S globulins (Onoue *et al.* 1965).

Fishman (1961) suggested that the 19S and 7S globulins are each synthesized by different plasma cells which are associated with the same antigen-engulfing macrophage. Nossal *et al.* (1964), on the other hand, postulated that during the course of the immune response, single maturing plasma cells switch from 19S to 7S globulin synthesis. Regardless of whether both 19S and 7S globulins are made by the same or different cells in a competent tissue, the mechanism responsible for the switch-over from production of 19S to 7S globulin in the antibody-synthesizing system as a whole is not yet clear. Some factors which influence this switch-over rate are as follow.

(a) The size of the antigen dose. When large doses of antigen are given to the antibody generating system, the time lapse between 19S and 7S globulin synthesis is decreased (Mellors & Korngold, 1963; Uhr & Finkelstein, 1963; Uhr, 1964; Svehag & Mandel, 1964), indicating that the antigen concentration activates the

switch-over mechanism. In cases of these large antigen doses, the 19S globulins are synthesized at a rapid rate, but are short lived while the 7S globulins increase at a slower rate but endure (Mellors & Korngold, 1963; Uhr & Finkelstein, 1963; Uhr, 1964; Svehag & Mandel, 1964).

(b) Use of Freund's complete adjuvant. Adjuvant, which has been said to cause non-specific antibody synthesis (Freund *et al.* 1948; John & Gersl, 1959; Uchitel & Khasman, 1965), actually causes an increase in 19S but not in 7S globulin synthesis (Benedict, 1965).

Both the aforementioned factors (a) and (b) would indicate that the 19S globulin is less specific than is the 7S globulin. Indeed, it has been suggested by Onoue (Onoue *et al.* 1965) that since the macroglobulin has six binding sites per molecule as opposed to two for the 7S globulin, it has greater (and perhaps more random) binding activity (Franklin & Kunkel, 1957; Korngold & Van Leeuwen, 1957; Müller-Eberhard, Kunkel & Franklin, 1956). Work with allergic individuals (Sehon, 1959) also indicates that the 19S Globulin is less specific than is the 7S globulin.

Unlike precipitating antibodies which are exclusively composed of 7S globulin, agglutinating antibodies may be either 19S or 7S globulins (Benedict, 1965; Benedict, Brown & Ayengar, 1962). Thus, it may be inferred from the previous discussion of specificity that precipitating antibodies are always specific, while agglutinating antibodies may be either specific or non-specific depending on whether they are composed of 19S (non-specific) or 7S (specific) globulins.

Since in the experiments reported here we used large doses of antigen in Freund's complete adjuvant, it would be reasonable that, at the time of the first antiserum collection (3 weeks after the first injection), both 19S and 7S globulins were present in the antiserum, the former in high concentration and the latter in low concentration (since it was already 'late' with respect to generation of the first antibodies). Since precipitins are present only in the 7S globulin fraction (Benedict, 1965) and because the 7S globulin is specific (Benedict, 1965), all precipitins present when agglutinating titres are low should be specific, as indeed we have found them to be (Vitetta & Guttman, 1967). Unlike precipitins, agglutinins are present in both the 19S and 7S fractions (Benedict, 1965). At low agglutinating titres, the concentration of 19S globulin should be higher than that of the 7S globulin, thus accounting for presence of non-specific agglutinins (Tables 2 and 3a). As the titre increases, the 7S globulin concentration should increase (Benedict, 1965; Daniel, 1965; Mellors & Korngold, 1963), accounting for the more specific agglutinating cross-reactions which were observed (Table 2). By the time peak titre was reached, 19S globulin synthesis should have ceased and 7S globulins exclusively produced (Benedict, 1965; Daniel, 1965; Mellors & Korngold, 1963), thus accounting for the high specificity of the cross-reactions (Table 3b). Our preliminary results do indeed show such relationships between 19S and 7S globulins. The fading or complete disappearance of the precipitating reaction at high agglutinating titre (Table 1) might be due to the masking of precipitins by non-precipitating antibody in the same globulin fraction (Gray, 1964). Accordingly, the detection of precipitins would depend on the ratio of precipitating to agglutinating antibody in the 7S globulin fraction at any particular agglutinating titre. At plateau and post-peak titres, despite the changes in homologous titre (slow-down of 7S globulin synthesis) the percentage cross-reaction should remain fairly constant, as indeed was observed (Table 3b).

We have thus shown the importance of the use of high-titre serum which is at peak or post-peak homologous agglutinating titre for the determination of specific immunological affinities by means of agglutinating cross-reactions. It is therefore probable that earlier disagreements among different workers (Clark, 1958; McGhee & Hanson, 1963; Noguchi, 1926) about immunological affinities within the genus Trypanosomatidae may have been due to inadvertent failure to select peak or post-peak titre antisera for the most specific cross-reaction determinations.

This work was aided by grant AI-06530 from the U.S. Public Health Service.

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