Folia Microbiol. 17, 105—111 (1972)

The Dynamics of the Formation of Virus-neutralizing, Complement-fixing and Immunofluorescent Antibodies to the Rabies Virus in Guinea Pigs

O. PROCHÁZKA, V. HRONOVSKÝ and H. DUBANSKÁ

Military Institute of Hygiene, Epidemiology and Microbiology, Prague 6

Received November 7, 1969
Revised version July 6, 1971

ABSTRACT. The aim of the present work was to establish the dynamics of the formation of virus-neutralizing (VN), complement-fixing (CF) and immunofluorescent (IF) antibodies in guinea pig antisera or fractions (IgM, IgG, 7S γ-2 7S γ-1 and F-fraction) obtained by gel filtration on a G-200 Sephadex column, or by chromatography on DEAE cellulose. It was shown that (1) there exists a correlation between the development and titres of VN and IF antibodies. This correlation was observed in both whole serum and its fractions IgG, 7S γ-2 and 7S γ-1 during the whole experimental period. (2) The formation of CF antibodies followed a different pattern in comparison with VN and IF antibodies. (3) VN, IF and CF antibodies were found to be bound to the IgG fraction, and most of the activity was found in subfraction 7S γ-2. The subfraction 7S γ-1 possessed approximately one half of the activity of fraction 7S γ-2 as regards VN and IF antibodies, and almost no activity of CF antibodies. (4) Both VN and CF antibodies, present in the IgM fraction, reached the maximum in the second blood sample, i.e. 14 days after the first dose of virulent virus. In the further course of the antibody response in guinea pigs, the curve of VN, IF and CF antibodies showed a reversed trend in the whole serum rather than in the IgM fraction.

The immunofluorescent method has been recently used on a large scale for the detection of the rabies virus in tissues of infected animals (Goldwasser and Kissling, 1958). Both the direct and indirect immunofluorescent method and the complement technique were used by these authors for the detection of the rabies virus. A number of authors stressed the advantages of the immunofluorescent method for the diagnostic purposes in the detection of the rabies virus (McQueen *et al.*, 1960; Etchebarne *et al.*, 1960; Topleninova, 1961; Klyueva *et al.*. 1966). Goldwasser and Kimron (1960) were able to detect the rabies antigen in the course of the incubation period, 1—2 days before the occurrence of Negri inclusions, stained according to Sellers. The crucial point that contributed to the decision whether to use this method for diagnosis, was a significant correlation between the results obtained by immunofluorescence and biological tests (Thomas *et al.*, 1963).

In the present work, several factors were taken into consideration: the above mentioned advantages of the immunofluorescent method; the well-established fact that the shift in the antibody activity takes place during the antibody response and, finally, that guinea pig immunoglobulin can be further separated chromatographically into two subclasses, having the 7S sedimentation coefficient and being different in terms of electrophoretic mobility in the starch gel block (Benacerraf *et al.*, 1963) and biological properties (i.e. passive cutaneous anaphylaxis, cytotoxicity). Among the differences in biological properties existing between these two subclasses, is the ability or inability to fix the complement and the different antigenic structure (Cohen and Porter, 1964; Bloch *et al.*, 1963; Ovary *et al.*, 1963).

In the present work, the antibody activity to the rabies virus was studied in the course of antibody formation, using complement fixation, virus neutralization and immunofluorescence with either whole or fractionated serum.

MATERIALS AND METHODS

The preparation of guinea pig antisera to the rabies virus. Guinea pigs were immunized according to our own immunization schedule, based on injection of two fixed strains of rabies virus with low and high virulence. Guinea pigs were injected with two preimmunizing doses of chicken embryonal suspensions of the avirulent strain HEP Flury ($10^{5.4}$ and $10^{5.5}$ LD_{50} in 1 ml), followed by five increasing doses of guinea pig brain suspension of the CVS strain (Challenge Virus Standard), starting with $10^{6.0}$ up to $10^{8.0}$ of mouse intracerebral LD_{50} in 1 ml. Live viral particles were injected together with a 30% lipoid adjuvant (sterile mixture of paraffin and lanolin, 3 : 1). 1 ml of this mixture was injected intraperitoneally per each injection in weekly intervals. The last immunizing dose of CVS virus was injected after an interval of two weeks.

Blood samples were withdrawn by cardiac puncture 2,4 and 7 weeks after the beginning of immunization. The last sample was taken two weeks after the last injection of CVS virus. Each sample was taken from 20 guinea pigs. The serum samples were frozen and kept at −20° C; some serum samples or fractions were tested immediately after withdrawal.

Determination of the virus-neutralizing activity (VN) in sera. The VN activity was tested using the standard VN test in mice (Johnson, 1964). Two-fold dilutions of non-inactivated sera (fractions) were mixed with a constant dose (100−300 LD_{50}) of CVS virus of a known titre. The mixtures of virus and serum (fractions) were preincubated for 90 min at 37° C and 30 min at 4° C prior to injection into mice.

Determination of complement-fixing (CF) activity in sera. Antigen for the CF test was prepared using our own modification of the original method of Ando (1953) and Schindler (1962). A 25% weight suspension was prepared from the brains of mice infected with the CVS strain and killed at the peak of infection. This suspension was submitted to two cycles of freezing and thawing, followed by elution (2 h at 25°C) and centrifugation. Supernatant was inactivated for 4 h at 56° C and then centrifuged at 6,000 r.p.m. The resulting supernatant was kept in glass ampoules on dry ice. A 1 : 16 dilution, established by previous box titration, was used in the CF test. The titres of antigen were found to be $10^{8.0}$ before inactivation, and less then $10^{0.6}$ LD_{50} in 0.03 ml after inactivation. The control CF antigen was prepared from brains of normal mice by the same procedure. No significant anticomplementary activity was found in both, i.e. control and infection antigens.

The "refrigerator method" was used for titration of CF antibodies, using 2 units of complement, 2 units of haemolysin and a 2% suspension of fresh sheep erythrocytes. The total volume of the reaction mixture was 0.5 ml.

With respect to the fact that most sera and fractions possessed an anticomplementary activity, all samples to be tested were centrifuged for 5 min at 30,000 r.p.m. and heat-inactivated for 20 min at 60° C. This procedure successfully removed the anticomplementary activity from all samples with the exception of the 7S γ-2 fraction and IgM, in which the anticomplementary activity could not be removed completely.

Separation of fractions of guinea pig antisera. First of all, the IgM and IgG immunoglobulins were separated using the Sephadex G-200 gel filtration (0.1 M tris-HCl buffer with 1.0 M NaCl, pH 8.0). The IgG fraction was then concentrated using carbowax, dialysed against a 0.035 M phosphate buffer, pH 7.5, and fractionated on a DEAE cellulose column according to the method of Reisfeld and Hyslop (1966).

The first peak was eluted by means of the initial buffer; the second, and starting with the second blood sample, even the third peak, was obtained by the final elution solution (0.5M tris—0.5M H_3PO_4, pH 3.7).

The presence of immunoglobulins in individual fractions was tested by electrophoresis in agar and by immunoelectrophoresis. The volume of all fractions was adjusted to the half volume of the original serum sample.

Immunofluorescent test (IF). The determination of the IF activity in antisera and fractions was based on the establishment of fluorescence staining titres by indirect method in a series of imprints of brains of mice infected intracerebrally with the rabies virus, forming large Negri inclusions. Two-fold dilutions of native sera or fractions were mixed with the supernatants of the 20% mice brain suspensions. Staining was performed using the FITC conjugate of pig anti-guinea pig serum (Institute of Sera and Vaccines, Prague), diluted 1 : 2. The highest dilution of antiserum (or fraction), yielding the intensity of the specific reaction denoted by one cross (i.e. clearly specifically stained antigen without brilliance), was taken as a staining titre.

The preparations were examined using the ML-2 fluorescent microscope (made in the U.S.S.R.), equipped with the mercury vapour lamp HBO-200 and appropriate filters. All comparative examinations were carried out using the ×40 objective (water immersion).

RESULTS

Separation of immunoglobulins. The crucial point appeared to be the separation of immunoglobulins in such a way that would exclude the presence of two immunoglobulins in one subfraction. Plate 1 depicts both photographically and schematically the results of separation of IgG immunoglobulins, using chromatography on DEAE cellulose. The 7S γ-2 fraction, that was eluted from the DEAE cellulose column by the initial buffer, did not contain any admixtures. The 7S γ-1 fraction contained a certain amount of impurities; however, no immunoglobulins were found. The F fraction (i.e. the third peak of the elution curve, obtained by the final elution buffer) contained only traces of 7S γ-1, and no other immunoglobulins were present.

The titration of antibody activity in sera and fractions. Table I shows relatively low titres of VN and CF antibodies found in the first sample, i.e. 7 days after the injection of the strain HEP Flury. However, if one examines closer the VN and CF titres, then a partial preponderance of VN antibodies over CF antibodies becomes evident. The immunofluorescence activity in sera was at that time higher than the titres of VN antibodies.

A significant increase in the titres of VN and CF antibodies was found after the injection of the virulent strain (i.e. in the 2nd sample). At the same time, even the immunofluorescent capacity in these sera increased. The VN and IF titres continued to increase until the 4th sample.

The IgM fraction. Relatively high titres of VN antibodies, i.e. maximum titres for the IgM fraction, were found in the IgM fraction of antisera obtained from the second sample. No CF antibodies were detected in this sample. In the third and fourth sample, a decrease in titres of VN antibodies was observed in this fraction. CF antibodies remained at the minimum level (1 : 2) throughout the whole experiment. Maximum immunofluorescent activity was reached in the IgM fraction in the second sample, and then the titre of these antibodies gradually decreased.

The IgG fraction. The majority of VN antibodies of the original serum was found in this fraction. The VN antibody titres had an increasing tendency from the first

TABLE I. The dynamics of development of immunofluorescent (IF), virus-neutralizing (VN) and complement-fixing (CF) antibodies in guinea pigs immunized with the rabies virus. The withdrawal of blood samples: (1) 2 weeks; (2) 4 weeks; (3) 7 weeks (4) 9 weeks after the beginning of immunization. The titres are expressed as reciprocal values of the highest dilution of antiserum (fraction) giving a positive reaction.

Serum (fraction)	Sample											
	IF				VN				KF			
	1	2	3	4	1	2	3	4	1	2	3	4
Original serum	64	256	1 024	2 048	18	370	8 310	32 760	2	64	128	128
Whole IgG	16	128	512	1 024	6	315	5 620	39 720	2	64	128	128
7S γ-2	16	32	256	256	3	75	2 590	3 340	2	32	32	32
7S γ-1	4	8	128	128	6	64	1 170	708	4	2	2	2
F	NT	4	4	4	NT	13	21	28	NT	2	2	2
IgM	2	16	8	4	3	140	35	32	2	2	2	2

NT = not tested

towards the fourth sample. In the first sample, the titres were at a very low level which corresponds to the overall low level of these antibodies in whole serum. Following the injection of two doses of virulent virus, the titre of VN antibodies grew in the IgG fraction as well as in the IgM fraction, i.e. approximately fifty times. From the third sample on, a profound difference in the dynamics of formation of VN antibodies in the IgG and IgM fractions took place.

The CF antibodies started to occur in the second sample, and no further changes took place in the fourth sample. The IgG fraction contained the total activity of CF antibodies of the original serum.

Antibodies, playing a role in the indirect immunofluorescence reaction, were also localised primarily in the IgG fraction. The increasing tendency of IF antibodies was found throughout the whole experimental period. The titres of these antibodies slightly preceded the titres of VN antibodies; however, towards the end of the experimental period they were at a much lower level.

The 7S γ-2 fraction. The fractionation of IgG immunoglobulin into further subfractions resulted in the separation of antibody activities: CF antibodies were completely localised in the γ-2 fraction; however, their titres never reached the values present in the whole IgG fraction (one half or one fourth of the original activity). On the other hand, as regards VN and IF antibodies, only part of the original activity of the IgG fraction was found in the γ-2 subfraction. A steady increase of both VN and IF antibodies was found between the first and last sample. A delay in the onset of VN antibodies was found when compared with IF antibodies in the first sample. If one takes the titres in the fourth sample as 100%, then a delay in VN titres could be found even in the second sample (2.25% for VN antibodies, 12.5% for IF antibodies). The growth of VN titres between the third and fourth sample in the γ-2 subfraction was much lower than in the whole IgG fraction.

The 7S γ-1 fraction. The second protein peak, eluted from the DEAE cellulose column by the tris-phosphoric acid buffer, did not contain CF antibodies. However, both VN and IF antibody activity was found. In both cases the activity was lower than in the γ-2 subfraction. As regards IF antibody activity, the maximum titres were reached in the third and fourth sample, as in the γ-2 fractions. As to VN antibodies, the peak titre was found in the third sample whereas a slight decline was observed in the fourth sample.

The F fraction. The third protein peak, eluted from the DEAE cellulose column by the tris-phosphoric acid buffer (pH 3.7), was called the F fraction. Both CF and IF activity were minimal in this fraction. In addition, even VN antibodies were very low throughout the whole experimental period. The low antibody activity found in this fraction probably could be attributed to a slight contamination by 7S γ-1 fraction rather than to the activity of the F fraction itself.

DISCUSSION

The dynamic of conversion of IgM to IgG immunoglobulin during antibody synthesis has been studied by a number of authors (Stelos and Talmage, 1957; Pike and Schulze, 1961; Bauer and Stavitsky, 1961; Uhr and Finkelstein, 1963; Svehag and Mandel, 1964; Rosenquist and Campbell, 1966). Hampar *et al.* (1968) observed the heterogeneity in properties of rabbit IgM and IgG VN antibodies to herpes simplex virus with respect to the complement fixation requirements. In addition, the differences in titres of VN antibodies in both main immunoglobulin classes, were reported by these authors in early and hyperimmune sera. From their work it further follows that VN antibodies in early sera (i.e. 7 days after immuniza-

tion), belonged to the IgM class and that they require the presence of complement, whereas in hyperimmune sera, these antibodies were primarily concentrated in the IgG fraction and did not require the presence of complement. Similarly Bellanti *et al.* (1965), who studied the character of antibodies in experimental arbovirus infection in guinea pigs, detected the VN and haemagglutination-inhibitory antibodies in antisera earlier than CF antibodies. The early haemagglutination-inhibiting and VN antibodies (i.e. 7—10 days after the beginning of immunization), were of the IgM nature, whereas CF antibodies belonged to IgG globulins. From the 14th day on, both haemagglutination-inhibiting and VN antibodies occurred even in IgG immunoglobulins. After a four months period, the total antibody activity was present in the IgG fraction. On the other hand, however, Graves *et al.* (1964) were not able to detect CF antibodies before the 16th day after the beginning of immunization.

In our experiments, the immunization with the avirulent HEP Flury virus resulted in very low titres of VN and CF antibodies. Only the injection of a virulent strain resulted in the production of high titres of antibodies. The VN antibody activity at the time of the second sample (i.e. after two injections of the virulent virus), was distributed between both IgM and IgG fractions. The VN antibody activity was at that time higher in the IgG fraction than in IgM globulin. The CF antibodies appeared in the second sample for the first time and they were localised in the IgG fraction only; particularly the subfraction 7S γ-2 contained the CF activity.

The results of our experiments are in agreement with the above mentioned authors, particularly the finding that VN antibodies from the early phase of immunization were present in the IgM fraction. In addition, we could also confirm the results of Graves *et al.* (1964). Furthermore, in agreement with the finding of Benacerraf *et al.* (1963) and Bloch *et al.* (1963), we found the total CF antibody activity in the 7S γ-2 fraction. No activity was found in the IgM or 7S γ-1 fraction. Finally, in agreement with these authors, the VN activity was found in the 7S γ-2 and 7S γ-1 fractions in the second, third and fourth sample. The IgM fraction possessed a low activity in the indirect immunofluorescence reaction. The highest titres of IF antibodies were obtained in the second sample followed by a decrease in antibody activity. This is in complete correlation with the occurrence of VN antibodies in this fraction. The greatest portion of IF activity was present in the IgG fraction, particularly in the 7S γ-2 subfraction. This finding corresponds to high VN antibody titres in this fraction as compared with 7S γ-1. Mutually independent changes in titres of IF and CF antibodies were found. This finding was further supported by the absence of CF activity in the 7S γ-1 fraction, possessing IF properties. Certain differences between the development of IF and VN antibodies during the early phase of immunization and in the later samples are of interest. The fact that higher titres of IF antibodies than VN antibodies were found in the first sample and, on the contrary, high levels of VN antibodies were present in the first sample, could be possibly explained by a lower neutralizing activity of IgM antibodies in comparison with IgG antibodies, as shown by Svehag (1964) in a polio-virus model. With an increasing ratio of IgG antibodies the virus-neutralizing activity of serum grew faster than its IF properties.

The plate will be found at the end of the issue.

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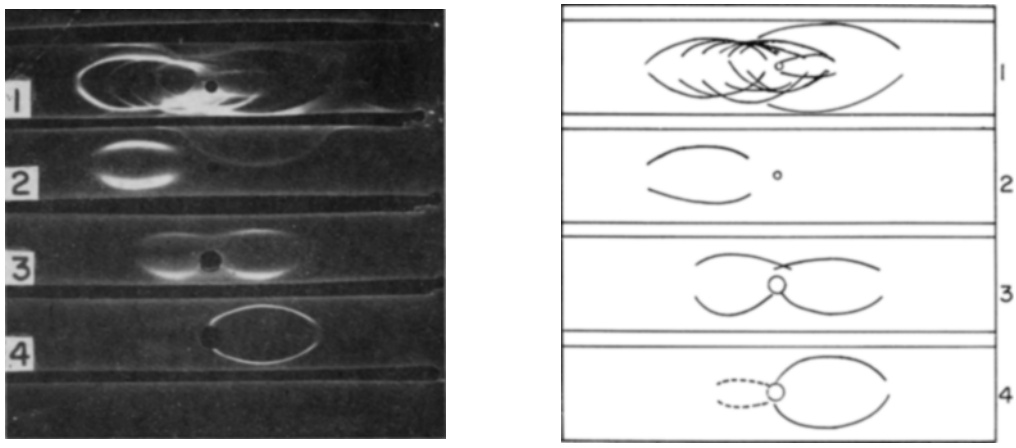

PLATE 1. Figure (left) and scheme (right) depicts the immunoelectrophoretic analysis of subfractions 7S γ-2 (2). 7S γ-1 (3) and F fraction (4), obtained by chromatography of IgG immunoglobulin on DEAE cellulose. For comparison, (1) shows a fraction of guinea pig serum obtained by salting out with ammonium sulphate (40% saturation). The wells contain fractions, the troughs pig antirabbit serum (against the whole serum).