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The Immunodepressive Effect of Friend Virus

II. REDUCTION OF SPLENIC HAEMOLYSIN-PRODUCING CELLS IN PRIMARY AND SECONDARY RESPONSES

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Summary. The effect of Friend virus on the response of Balb/c mice to sheep red cells, as measured by the numbers of haemolytic plaque forming cells in the spleen, was investigated. Virus injected with or before the antigen reduced the numbers of IgM and IgG antibody-producers, the latter more so than the former. Both the primary and the secondary responses were affected. Some possible mechanisms of action of the virus on immune responses are discussed.

INTRODUCTION

Friend virus (FV), injected before sheep erythrocytes (RBC), was found to depress the primary and secondary immune responses to this antigen as judged by haemagglutinin titres and the number of haemolytic plaque forming cells (PFC) (Jerne, Nordin and Henry, 1963) in the spleen (Salaman and Wedderburn, 1965, 1966, 1968; Odaka, Ishii, Yamaura and Yamamoto, 1966).

Rauscher virus (RV), an agent very similar to FV in its pathological effects, also depresses antibody responses to both RBC and bovine serum albumin (Salaman and Wedderburn, 1965; Salaman, 1968; Siegel and Morton, 1966a, b; Gelzer and Dietrich, 1968). Cremer and her colleagues (Cremer, Taylor and Hagens, 1966; Cremer, 1967) have reported that rats, infected when newborn with Moloney virus, show a depressed response to RBC both in the leukaemic and the pre-leukaemic phases, and also a gradually increasing deficiency in serum γ-globulins after the age of 4 weeks. Metcalf and Moulds (1967) found that serum antibody responses of AKR mice, a high-leukaemic strain, were depressed in leukaemic though not in pre-leukaemic animals, and these results correspond to our findings in mice infected when newborn with Moloney virus, which induces a lymphoid leukaemia very similar to the spontaneous disease in AKR mice (Salaman and Wedderburn, 1966, and unpublished).

Our object in the present work has been to follow the course of depression of the primary and secondary immune responses to RBC in mice infected with FV by counting haemolytic plaque forming spleen cells at intervals after an injection of sheep RBC.

Direct plaques, as detected by the original technique (Jerne *et al.*, 1963), which are probably due to 19S antibody forming cells, and 'developed' plaques (Šterzl and Říha, 1965; Dresser and Wortis, 1965), probably due to 7S antibody forming cells, were separately estimated.

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MATERIALS AND METHODS

Animals

(1) Balb/c mice of either sex, bred in the department by brother–sister mating, and aged 8–13 weeks, were used. In any one experiment mice were of the same sex and did not differ in age by more than 1 week.

(2) C57 Black mice were bred as above in the animal house of the London Hospital Medical College.

Viruses

Friend virus. Friend Virus (FV) (Friend, 1957) was sent by Dr Charlotte Friend to the Chester Beatty Research Institute in 1959 and passed in Swiss or DBA mice. Since 1962 it has been passed in Balb/c mice in the Cancer Research Department of the London Hospital Medical College, where it has been freed from the lactate dehydrogenase elevating virus of Riley, Lilly, Huerto and Bardell, 1960 (Mahy, Rowson, Salaman and Parr, 1964). This strain of virus produces thrombocytopenia and anaemia as well as splenomegaly in Balb/c mice.

Moloney leukaemia virus (MLV). MLV (Moloney, 1960) was kindly supplied by Dr J. B. Moloney in 1960, and subsequently passed through mice and rats in this laboratory. This strain is free of Riley virus.

Suspension, inoculation and titration of virus

Twenty per cent spleen homogenates from infected mice were made in Dulbecco's phosphate buffered saline solution A (PBS) (Dulbecco and Vogt, 1954) containing 0·1 per cent gelatin, spun first for 5 minutes at 1000 *g* and then for 20 minutes at 8000 *g* at +4°. The final supernatant was stored at −60°. Occasionally citrated plasma was used as inoculum. Mice were inoculated intravenously via the orbital sinus. FV batches were titrated by injecting 0·1 ml volumes of successive ten-fold dilutions into groups of three or four mice and weighing their spleens 3 weeks later. Spleen weights of 0·35 g or more were taken as significant evidence of infection. All the batches of FV in the experiments described contained 10^3–10^4 intravenous ID_{50}/0·1 ml. Titration using intraperitoneal inoculation gave titres which were lower by a factor of about 10.

Sheep red blood cells (RBC)

These were obtained from Burroughs Wellcome and Co., suspended in Alsever's solution. They were washed twice in citrated PBS and twice in saline, and made up to the required concentration based on packed cell volume. A volume of 0·1 ml of a 10 per cent suspension contained approximately $2{\cdot}5 \times 10^8$ RBC.

All injections of RBC were made intravenously via the orbital sinus. Because successive batches of RBC are not taken from the same animal (Burroughs Wellcome, personal communication), and also in order to eliminate the possible effect of slight variations in the number of RBC injected, contemporary controls were run with every experiment. The day of RBC injection was reckoned as day 0, and intervals before and after this as negative and positive, respectively.

Guinea-pig complement

Burroughs Wellcome azide-preserved complement (Richardson, 1941) was used, and diluted until equivalent to 1:10 fresh guinea-pig serum.

Eagle's medium

This is 'Eagle's medium for use with suspended cells' from Glaxo Ltd, which is Eagle's medium (Eagle, 1959), containing 10 per cent tryptose phosphate broth, and also 1155 mg lactalbumin hydrolysate per litre and 100 units each of penicillin, streptomycin and polymyxin per litre.

Haemolytic plaque test

Estimation of direct plaques. The method was based on that of Jerne *et al.* (1963) with modifications. Base layers of 1·4 per cent Noble agar (Difco Laboratories) in complete PBS (containing calcium and magnesium), to which were added 0·3 mg/ml of DEAE dextran (Pharmacia), were made in 8-cm diameter Petri dishes. Top layers consisting of 0·7 per cent agar in Eagle's medium containing 0·15 mg/ml of DEAE dextran, to which were added 0·1 ml of 20 per cent RBC and 0·1 or 0·2 ml of spleen cell suspensions in Eagle's medium, were poured onto the base layers and allowed to cool. The dishes were incubated for 1 hour at 37°. To each dish was added 1·5 ml of 1 : 10 guinea-pig complement and they were incubated for a further 45 minutes. Plaques were counted by transmitted light using a hand lens of 10 cm focal distance.

Estimation of developed plaques. These plaques were estimated by a modification of the method described by Wortis, Taylor and Dresser (1966). A rabbit anti-mouse γ-globulin serum which reacted with all classes of mouse immunoglobulins, heated to 56° for 30 minutes and absorbed with washed sheep RBC, was kindly supplied by Dr Dresser. After titration this serum was used by incorporating it into the top layer of agar at a concentration of 1:3150. Allowance was made for the inhibitory effect on direct plaque production observed at this concentration, which appeared to be slightly less on normal than on infected spleen cells, viz. 15 per cent on normal cells and 23 per cent on infected cells. Various explanations could be advanced for this apparent difference; for example the uninfected spleen cells 2 days after RBC injection, used to determine the degree of inhibition of direct plaques, may in fact have included some developed plaque formers (although they are assumed not to do so at this stage in the response), while there may have been none among the infected spleen cells. In any case by using the different degrees of inhibition of direct plaques from infected and normal mice as they stand, the degree of inhibition of developed plaques caused by FV can only be underestimated.

Estimations of the numbers of nucleated cells per spleen

As early as 4 days after the inoculation of FV the spleen begins to enlarge and the total number of nucleated cells in the spleen begins to rise. This rise continues during the course of the disease. Most of the cells are red cell precursors, not belonging to the lymphoid series; consequently it is not meaningful to relate PFC to total nucleated cell count. For these reasons the plaque forming cell counts were expressed as PFC/spleen. If expressed as PFC/10^6 nucleated cells the counts would have shown a much greater degree of depression by FV, particularly in the late stages of the disease, than when expressed as PFC/spleen.

When spleens reach a weight of about 1·5 g or more they become increasingly necrotic, and contain large zones of haemorrhage. Also the proportion of viable cells, as judged by dye-exclusion, falls sharply. Such spleen suspensions are difficult to prepare. For these reasons we have tried to avoid the later stages of the disease as far as possible.

Estimation of uptake of RBC

^{51}Cr (Radiochemicals, Amersham, Bucks), in the form of a sodium chromate solution which contained 9·9 μg Cr/ml and had an activity of 1 mc/ml, was used to label RBC suspensions. A 50 per cent suspension of RBC was prepared in the usual way. Thirty to 40 μc of ^{51}Cr were added to 3 ml of RBC suspension, which was then left at room temperature for 1 hour. The RBC were then washed three times with saline. No radioactivity was detectable in the final washing. The required amount of RBC was then injected intravenously. Twenty-four hours later the mice were bled under ether anaesthesia from the

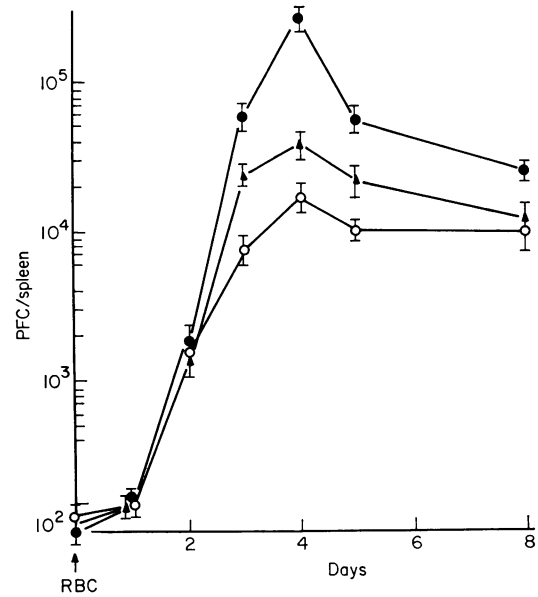

FIG. 1. Effect of FV, injected at various times before $2·5 \times 10^8$ RBC, on the direct PFC count per spleen. RBC injected on day 0 to all groups. ●, Normal mice; ▲, FV injected on day −2; ○, FV injected on day −4. Four mice per group. Limits represent ± 1 standard error of the geometric mean.

brachial artery, killed, and their spleens and livers weighed and transferred to plastic tubes. Counts per minute were then estimated on a Packard γ spectrophotometer, and the percentage of the original activity found in the spleens and livers calculated.

Statistical analysis

Two plates were used for each spleen suspension and the mean count taken. Three or four mice were used per group. The geometric means of the PFC/spleen were calculated, and are shown in the figures, together with limits of 1 standard error of the geometric mean. In experiments using ^{51}Cr, six mice per group were used and the standard errors of the mean counts calculated in the usual way.

RESULTS

EFFECT OF FV ON THE NUMBERS OF PFC PER SPLEEN AFTER A PRIMARY INJECTION OF RBC

FV was injected into groups of mice 7, 4 and 2 days before an injection of $2·5 \times 10^8$ RBC. Fig. 1 shows the number of PFC found at intervals after the RBC injection. The

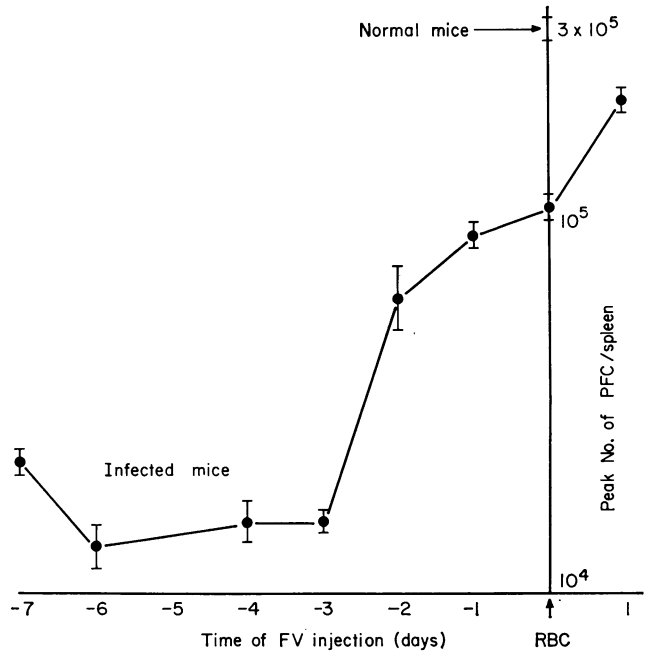

FIG. 2. Effect of FV, injected at various times relative to RBC, on the number of direct PFC 4 days after $2{\cdot}5 \times 10^8$ RBC, using four mice per group.

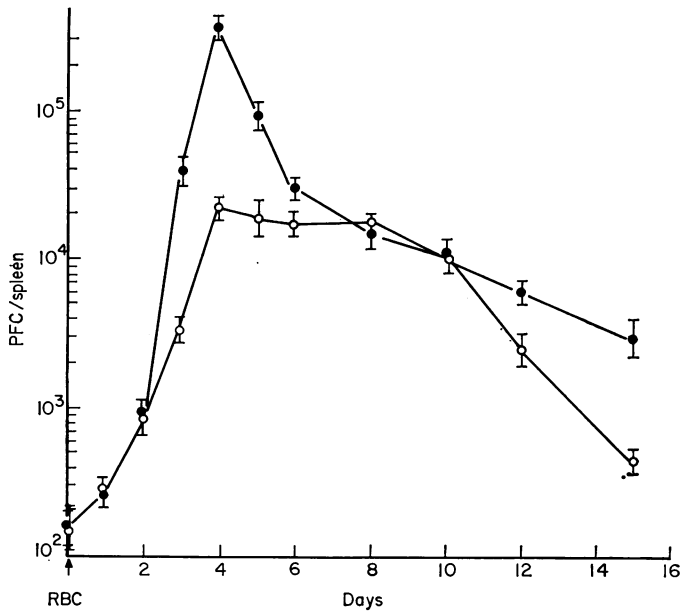

FIG. 3. Effect of FV 4 days before $2{\cdot}5 \times 10^7$ RBC on the number of direct PFC per spleen. ●, Normal mice; ○, FV day − 4. Four mice per group.

numbers of PFC in the spleens of mice which received FV 4 or 2 days before antigen are depressed, the former more than the latter. Animals which received FV 7 days before RBC gave results indistinguishable from those which received FV 4 days before RBC, and have been omitted. In both control and experimental animals PFC reached a peak on the 4th day after antigen: on this day there was an approximately sixteen-fold depression of PFC in those animals which had received FV 4 days before RBC.

Fig. 1 shows that the peak PFC count, in both normal and infected mice, is reached 4 days after a dose of $2{\cdot}5 \times 10^8$ RBC. The effect of giving virus at various times before and after this dose of RBC on the number of PFC per spleen present 4 days after RBC is shown

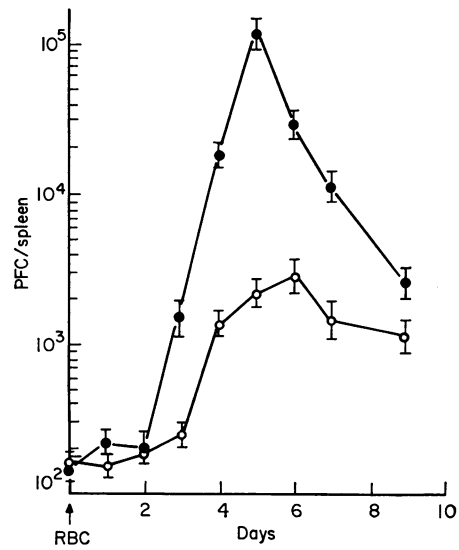

FIG. 4. Effect of FV 4 days before $2{\cdot}5 \times 10^6$ RBC on the direct PFC per spleen. ●, Normal mice; ○, FV day − 4. Four mice per group.

in Fig. 2. These results are similar to those previously obtained using 7th day haemagglutinin titres (Salaman and Wedderburn, 1966). When the virus was injected 1 day after RBC no significant depression was observed. A significant depression was obtained when the virus was given at the same time as the RBC. The earlier the virus was given the more marked was the depression, up to an interval of 3 or 4 days. Further increase of the interval did not cause any further increase in the degree of depression, unless the virus was given at such a time (about 10 days or more before the antigen) that determinations had to be carried out during the terminal stages of the disease. When this occurred, for example in the experiment shown in Fig. 3 where two groups of mice were followed for longer periods than usual, and spleen weights of up to 3 g were found, a further depression of the PFC per spleen of infected mice was observed.

EFFECT OF VARYING THE DOSE OF RBC

Fig. 1 showed the PFC response following injection of $2{\cdot}5 \times 10^8$ RBC into normal mice and mice infected with FV 4 days previously (top and bottom curves). Two other doses of RBC were tried, $2{\cdot}5 \times 10^7$ and $2{\cdot}5 \times 10^6$ RBC. The effect of FV injected 4 days before

these doses is shown in Figs. 3 and 4. Two and a half$\times 10^7$ RBCs produced similar results to $2{\cdot}5\times 10^8$ RBC (cf. Figs. 1 and 3). Two and a half$\times 10^6$ RBCs produced a rather lower peak PFC count, and this was delayed by 1 day, to day 5. The peak in infected mice was on day 6, though the counts on days 5 and 6 did not differ significantly. The degree of

TABLE 1

NUMBER OF PFC PER SPLEEN ON THE 2ND AND 3RD DAYS AFTER VARIOUS DOSES OF RBC

No. of RBCs injected	PFCs per spleen 2 days after RBCs			PFCs per spleen 3 days after RBCs		
	FV	Controls	*P**	FV	Controls	*P**
$2{\cdot}5\times 10^6$	358	334	>0·1	311	3,880	<0·01
$5{\cdot}0\times 10^6$	616	491	>0·1	659	7,960	<0·01
$1{\cdot}25\times 10^7$	880	941	>0·1	1,630	30,000	<0·01
$2{\cdot}5\times 10^7$	1,950	2,090	>0·1	4,710	49,300	<0·01
$2{\cdot}5\times 10^8$	2,520	2,935	>0·1	10,800	88,800	<0·01
$2{\cdot}5\times 10^9$	3,880	5,530	>0·1	12,800	84,000	<0·01

* Probability that the difference between treated and control animals could arise by chance.

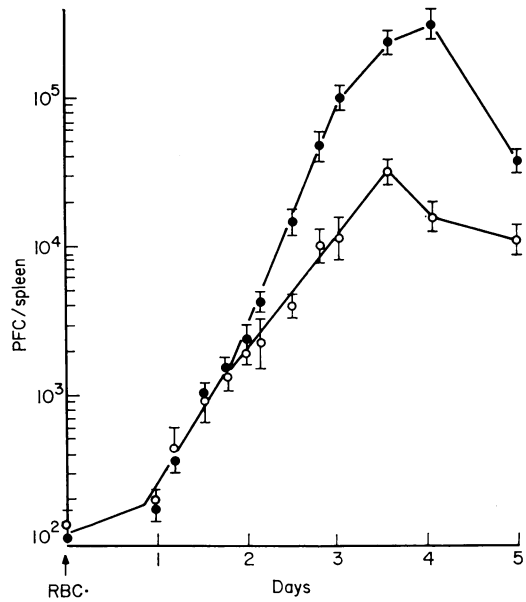

FIG. 5. Effect of FV 4 days before $2{\cdot}5\times 10^8$ RBC on the direct PFC per spleen determined at closely spaced intervals after RBC. ●, Normal mice; ○, FV day − 4. Four mice per group.

depression of the peak PFC count in this case was greater: 2000 PFC in infected spleens compared with 100,000 PFC in normal spleens, a fifty-fold depression. A similar effect of antigen dose on immune depression of another kind has been observed by Nettesheim, Makinodan and Williams (1967). These authors found that while spleen cells from normal RBC-primed donors transferred to lethally irradiated recipients together with RBC gave a near optimal secondary response to doses of 10^4 RBC upwards, similar cells from sublethally irradiated donors needed a dose of 10^8–10^9 RBC for an optimal response.

It can be seen in Figs. 1, 3 and 4 that while in each case the infected animals showed a depressed number of PFC on day 3, there was no significant depression on day 2 in spite of the fact that about 2000 PFC per spleen were present (see Fig. 1), a rise of fifteen- to twenty-fold above background. A further experiment showed that even when virus was given as long as 10 days before the antigen ($2{\cdot}5 \times 10^8$ RBC) there was still no significant depression on day 2.

An experiment in which the response during the first few days was further analysed by closely spaced estimates of PFC confirmed the absence of depression at 2 days (Fig. 5). It can be seen that the curves for normal and experimental animals did not begin to separate until after 48 hours.

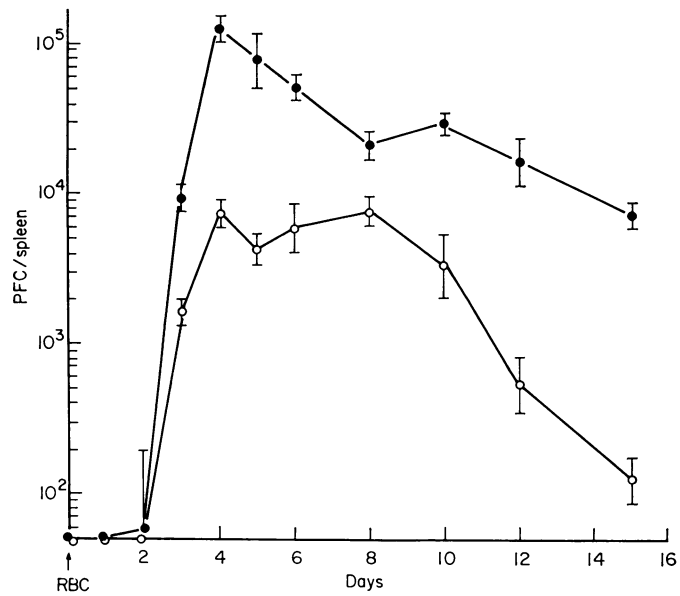

FIG. 6. Effect of FV 4 days before $2{\cdot}5 \times 10^7$ RBC on the developed plaques per spleen. Same mice as those used to determine values in Fig. 3. Points on the base line without limits represent values of less than 50 PFC/spleen. ●, Normal mice; ○, FV day − 4.

Table 1 shows the result of an experiment in which the PFC per spleen 2 and 3 days after RBC were determined over a 1000-fold range of antigen dose. This shows that a very large depression of PFC was obtained on day 3 at all doses of antigen, while on day 2 no significant depression was found at any dose. (Following the largest dose of antigen there was a depression of PFC count in infected mice on day 2, but this was not significant.)

EFFECT OF FV ON THE APPEARANCE OF IgG PRODUCING CELLS

It was of interest to discover whether FV infection affected the appearance of IgG as well as IgM producing cells. The great majority of PFC in the direct Jerne test are assumed to be IgM producers, since IgG antibody is only weakly haemolytic (Humphrey and Dourmashkin, 1965). By the use of a rabbit anti-mouse immunoglobulin serum, as described in 'Materials and methods', an estimate could be made of 'developed' plaques, i.e. those due to presumptive IgG producers. Fig. 6 shows such counts for the same

experiment from which direct plaques were plotted in Fig. 3, i.e. following a dose of $2{\cdot}5 \times 10^7$ RBC in normal and infected mice. The first significant rise in the developed plaque count occurred on day 3 in both normal and infected mice. In contrast to the direct plaque count, a depression in infected animals was evident from the time when the first increase in these plaques was noted.

EFFECT OF FV GIVEN 4 DAYS BEFORE A SECOND DOSE OF RBC ON THE SECONDARY RESPONSE

After some preliminary experiments in which various doses of RBC and various intervals between doses were tried, a regimen was devised in which a large secondary response

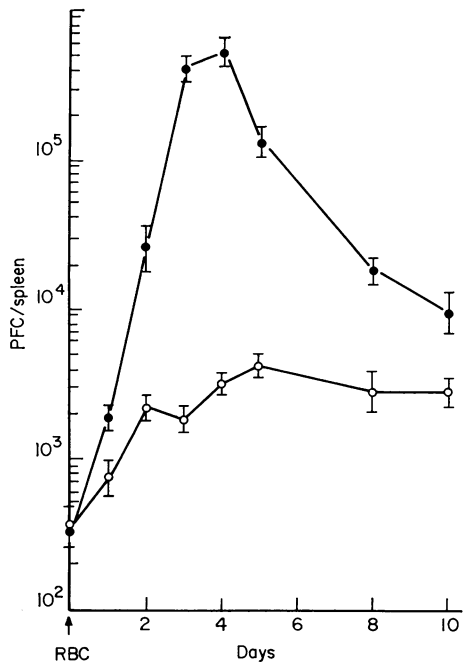

FIG. 7. Effect of FV 4 days before a second dose of RBC on the direct PFC per spleen. Primary dose $2{\cdot}5 \times 10^6$ RBC. Challenge dose 5 weeks later $2{\cdot}5 \times 10^8$ RBC. ●, Normal mice; ○, FV day − 4. Four mice per group.

was obtained using a primary dose of $2{\cdot}5 \times 10^6$ RBC followed 5 weeks later by a secondary dose of $2{\cdot}5 \times 10^8$ RBC (cf. Jílek and Šterzl, 1967; Biozzi, Stiffel, Mouton, Bouthillier and Decreuseford, 1968). It was decided to investigate the effect of FV given a short time before the second dose of RBC, so as not to complicate the results by adding the possible effects of infection on the events which occur early in the period between the priming and challenge doses.

Figs. 7 and 8 show the results of giving FV 4 days before the second dose of antigen, as measured by 'direct' and 'developed' plaque counts. A rise in direct PFC count was evident on day 1, at which time infected mice already showed a depression. Developed plaques first rose on day 2: at this time infected animals already had a smaller number of PFC. The degree of depression of the secondary response for both direct and developed

plaques was greater than that observed in a primary response to the dose ($2{\cdot}5 \times 10^8$ RBC) which was used for challenge.

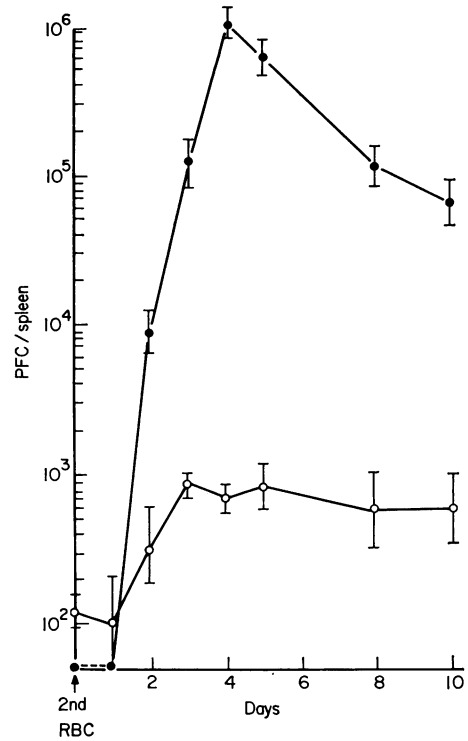

FIG. 8. Effect of FV 4 days before a second dose of RBC on the developed PFC per spleen. Same mice as those used to determine values in Fig. 7. ●, Normal mice; ○, FV day − 4. Points on the base line without limits represent values of less than 50 PFC/spleen.

TABLE 2

RECOVERY OF ^{51}Cr AFTER INJECTION OF LABELLED RBC

No. of ^{51}Cr labelled RBC injected	FV injection	^{51}Cr recovered		
		Per cent of total dose in the spleen	Per cent of total dose per 100 mg spleen (wet weight)	Per cent of total dose in the liver
$2{\cdot}5 \times 10^6$	FV on day − 4	5·80	3·43	79·7
$2{\cdot}5 \times 10^6$	Nil	4·63	3·65	88·3
$2{\cdot}5 \times 10^8$	FV on day − 4	8·73	4·90	58·6
$2{\cdot}5 \times 10^8$	Nil	9·94	6·89	57·4
$2{\cdot}5 \times 10^8$	FV on day − 11	4·37	0·44	78·6
$2{\cdot}5 \times 10^8$	Nil	8·84	5·55	57·5

ESTIMATION OF THE UPTAKE OF RBC BY INFECTED SPLEENS

Another possible way in which FV might produce immune depression is by decreasing the uptake of antigen by the spleen. It was therefore of interest to determine whether the

uptake of RBC by the spleen was altered in FV infected mice. For this purpose ^{51}Cr-labelled RBC were injected into normal and FV infected mice. The percentage of the administered ^{51}Cr recovered from their spleens and livers 24 hours later was determined.

Three groups of mice were injected with FV, the first two groups 4 days before $2{\cdot}5 \times 10^6$ and $2{\cdot}5 \times 10^8$ labelled RBC, respectively, and the third 11 days before $2{\cdot}5 \times 10^8$ labelled RBC. Comparable groups of uninfected mice received similar doses of labelled RBC. Mice of the first two groups had spleens weighing less than 0·2 g, and those of the third group had spleens weighing 0·9–1·2 g at the time of ^{51}Cr estimation. Spleen weights of normal adult Balb/c mice vary between 0·10 and 0·15 g. There was no alteration in liver weight of infected mice at this stage of the disease.

Table 2 shows the results of this experiment. Three measurements were compared: the percentage recovery of ^{51}Cr from the spleen, the percentage recovery per 100 mg of spleen, and the percentage recovery from the liver.

When the small dose of $2{\cdot}5 \times 10^6$ labelled RBC was given, a relatively higher proportion of the ^{51}Cr was found in the liver and a lower proportion in the spleen than after the larger dose of RBC, both in normal and infected mice. FV injection did not appear to affect the recovery from either organ after this dose of RBC. With $2{\cdot}5 \times 10^8$ RBC, however, mice which had been infected with FV 4 days before had rather less ^{51}Cr in their spleens, and when this was expressed as percentage ^{51}Cr recovered per 100 mg of spleen there was a significant difference between the values obtained for normal and infected spleens. Mice infected with FV 11 days before $2{\cdot}5 \times 10^8$ RBC had a lower percentage of ^{51}Cr in their spleens, which was significant when expressed either per spleen or per 100 mg of spleen tissue, and an increase in the percentage ^{51}Cr recovered from their livers.

It is evident that the degree of immune depression by FV is not proportional to the reduction in uptake of RBC by the spleen. For example, after $2{\cdot}5 \times 10^6$ RBC there is a maximal difference between normal and infected mice in PFC counts, but no difference in RBC uptake. The uptake of RBC may become an important factor in mice in the later stages of the disease, but the present experiments did not include these stages.

DISCUSSION

Early changes in the spleens of FV infected mice

The effect of FV on the immune response shows itself very early in the disease. The degree of depression of the primary response (serum haemagglutinin titres and number of haemolytic plaque forming cells in the spleen) reaches a maximum when the virus is injected 4 days before RBC. Increasing this interval (e.g. up to 10 days) does not significantly increase the depressive effect of the virus. This contrasts with the effect of MLV, which produces no depression of immune response at 4 days, but a gradually increasing depression from the appearance of leukaemia.

Four days after the injection of FV few histological or cytological changes in the spleen are visible with the light microscope (Metcalf, Furth and Buffett, 1959). However, there is good evidence that important and widespread changes have already occurred by this time.

After a large intravenous injection of FV (e.g. approximately 10^4 ID_{50}, as used in most of the experiments described above) the titre of virus recoverable from the spleen was found to rise sharply on the 3rd day, and on the 4th day reached a plateau of 10^3–10^4 ID_{50} per 0·1 ml of 20 per cent spleen extract. There was no further increase for

the following 4 days at least (Bendinelli, unpublished). Deaths among mice which have received this dose generally occur after day 19.

Silber, Goldstein, Berman, Decter and Friend (1967), using virus doses similar to ours, showed that uridine incorporation into RNA in spleen cells rose sharply between the 2nd and 4th days after intraperitoneal injection of FV, reaching a peak value of four times the normal. By the 6th day it had decreased to the normal value, and remained there in spite of the fact that the greatest increase in spleen weight occurred after the 6th day. Ribonuclease activity in the spleens of infected mice decreased to less than one-third of the normal by the 4th day, and declined still further during the later stages of the disease. These authors conclude that either the few morphologically abnormal cells present on the 4th day must be synthesizing RNA at an extremely high rate, or that many other cells must have a moderately increased rate of RNA synthesis. The latter possibility, they point out, is compatible with the observation that morphologically normal cells are able to support replication of FV (de Harven and Friend, 1960).

FV produces a marked alteration in the splenic cellular reaction to a powerful antigenic stimulus. Chan, Rancourt, Ceglowski and Friedman (1968) have recently shown that FV modifies the early changes in mouse spleen which follow intraperitoneal injection of a large dose of sheep RBC. Instead of the well-defined lymph follicles with active germinal centres, containing large numbers of mature plasma cells, which were seen in uninfected mice 4 days after RBC injection, in mice injected with FV 3–7 days before the antigen lymph follicles were ill-defined, and contained few plasma cells but many blast-like cells. Electronmicrographs showed virus particles in the cytoplasm of blast-like cells, but none were found in the rare plasma cells.

There is some evidence that splenic virus content at the time of antigen injection is not the only factor affecting the degree of immune depression. We are at present studying the effects on the immune response of different doses of FV given at different times before the antigen. Early results show that when different doses are injected on the same day the degree of immune depression is directly dependent on virus dose, but that the effects of smaller doses injected at longer intervals before the antigen cannot be explained in terms of splenic virus content at the time of antigen injection. The size of the virus inoculum as well as the splenic virus content attained appears to affect the result. Another possible complicating factor is the presence in our FV stocks of another virus, as recently demonstrated by our colleagues (Rowson and Parr, 1968), which causes minimal pathological changes but immunizes against FV infection, and has itself appreciable immunodepressive effect. The relations between these factors will be the subject of a separate report.

The possible role of antigenic competition and of neutralizing antibodies

FV stimulates the formation of neutralizing antibodies, which first become apparent at 2 weeks (Rowson and Gillespie, unpublished). However, Mathot, Rothen and Scher (1965), using an electroadsorption method, demonstrated antibodies to FV 2 days after infection, and these rose to a maximum in about 2 weeks. Judged by the dilution of serum which would give a positive reaction in their system, FV was a very weak antigen compared with eight arboviruses which they tested.

Albright and Makinodan (1965) and Radovich and Talmage (1967) have investigated the effect of an injection of one foreign species of erythrocytes on the antibody response to a second species injected at various intervals after the first. In the system used by the

former authors the total haemagglutinating antibody produced in a primary response by mice to rat RBC was measured over a period of 10 days. They found that the interfering effect of sheep RBC was greatest when given about 15 hours before the rat erythrocytes, and had no effect on a secondary response to this antigen.

Our results show that the effect of FV, as estimated in this way, is different from the effect of a second species of erythrocytes, both because of the different optimal interval for depression between FV and RBC, and because the secondary response is strongly depressed by the virus but not at all by the erythrocytes.

Our results correspond more closely to those of Radovich and Talmage (1967), who found that an injection of horse RBC given at various times before sheep RBC produced the greatest interference with the primary response to the latter antigen, as measured by the 4th day PFC per spleen against sheep RBC, when there was an interval of 4 days between the two injections. Results of other experiments in which spleen cells were transferred to irradiated recipients led them to conclude that the inhibition was mediated by a humoral feedback; i.e. that substances liberated during the first immune response were responsible for the inhibition of the second.

In order to examine further the possibility of antigenic competition we used a system very susceptible to possible depressive effects: the response to $2{\cdot}5 \times 10^6$ RBC. In this system we found no depressive effect by a large dose of formolized FV (non-infective but antigenic, Friend, 1959), nor by a large dose of MLV, although the latter depressed the response 4–6 months later, when the mice became leukaemic. We also showed that a large dose of FV had no effect on PFC counts after RBC injection in C57 Black mice, which are very resistant to FV but are good antibody producers (Brooke, 1965; Andersson, Wigzell and Klein, 1967).

On the other hand a dose of about 15 ID_{50} of FV given 4 days before RBC produces a significant depression of the response; this is a very small amount of antigen compared with the dose of formolized FV which was used. Though the small dose of live virus will of course proliferate, the virus recoverable from the spleen 4 days after such a dose is barely detectable by our methods. We, therefore, agree with Odaka *et al.* (1966) that live FV is necessary for an immunodepressive effect, although we have not ruled out a slight additional effect of antigenic competition.

The virus-sensitive cell

Our experiments using labelled RBC showed that the effect of FV cannot be accounted for by a reduction in the uptake of RBC. On the other hand this may become an additional factor when mice with spleens of 1 g or more are given RBC injections, and may explain results which we obtained using mice in the late stages of the disease (Salaman and Wedderburn, unpublished) when degrees of immune depression greater than those described in this paper were found.

However, moderate doses of X-rays, for example, do not affect uptake of antigen but appear to alter its processing in some way (Gallily and Feldman, 1967; Donaldson, Marcus, Gyi and Perkins, 1956; Gordon, Cooper and Miller, 1955). Alteration of the ratio of different types of phagocytosing cell can also affect the subsequent antigenic efficacy of ingested antigen (Cohn, 1962). Interference of FV with RBC digestion or with the processing of the antigen may possibly occur, as FV has been shown to multiply in macrophages (Odaka and Köhler, 1965). But a simple lack of antigen, processed or otherwise, cannot explain the immune depression by FV, because the course of develop-

ment of PFC in the spleens of infected mice after a large dose of RBC cannot be matched by injecting smaller doses into normal mice.

A very recent report by Chan *et al.* (1968) describes the effect on haemolysin titres and PFC counts of an intraperitoneal injection of FV given at various times relative to a large intraperitoneal dose of RBC. Their results differ from ours in several ways, and it is difficult to say how far the difference can be accounted for by differences in injection routes and technique. In their system, for example, there was a depression of PFC 2 days after RBC in mice given FV 0–12 days before the antigen, and the degree of PFC depression at all stages of the reaction increased as the interval between FV and RBC injection was lengthened. There was proportionally much less depression of haemolysin titres than of PFC counts: they record, for example, a peak PFC depression of 55-fold with a six-fold depression of peak haemolysin titre. In our system peak PFC counts and peak haemolysin titres were depressed by about the same factor. The explanation of these differences must be left for further investigation.

Siegel and Morton (1966a, b) studied immune depression by Rauscher virus. On the basis of their results they put forward the theory that this virus competes with RBC not as an antigen, but as an infective cell-transforming agent which can act on uncommitted stem cells; that these stem cells can respond either to the virus or to the antigenic stimulus of the RBC but not to both; and that committed cells, whether producing antibody, or merely possessing the information to do so, are not affected. Our results support part but not all of this hypothesis. Virus given several days after RBC did not depress the plateau level of haemagglutinin, suggesting that active antibody production by committed cells is not affected, but when virus was given 4 days before a second injection of RBC the secondary response (both IgM and IgG) was severely depressed, suggesting that the reaction of committed cells to a second stimulus *is* affected. Furthermore, we have no evidence that committed cells, whether producing antibody or not, are unable to support viral replication. We found that RBC given either 4 days or 1 hour before FV actually slightly increased the apparent titre of the virus, as estimated by splenomegaly at 3 weeks. Siegel and Morton (1966a) found a similar effect of bovine serum albumin in Freund's adjuvant on the course of the disease produced by Rauscher virus. These effects could be due either to antigenic competition causing a depression of the amount of neutralizing antibody formed to the virus, or to cell proliferation caused by the injections of antigen providing more cells available for infection.

Virus sensitive and virus resistant parts of the immune response

It has been shown above that in the primary response of a normal mouse to $2{\cdot}5 \times 10^7$ RBC or more, 2000 or more IgM-forming PFC appeared in the first 2 days, while $2{\cdot}5 \times 10^6$ RBC produced only about 300–400, and that neither value was significantly depressed in the infected mouse. By the 3rd day, the larger dose produced 50,000 or more PFC while the small dose produced 3000–4000, and both values were depressed in the infected mouse. We may conclude, therefore, that the response to a small dose of RBC is not just the delayed equivalent of that to the larger dose, because the onset of susceptibility to depression by FV is not delayed: both, at their respective levels, are resistant on the 2nd and susceptible on the 3rd day.

It therefore seems possible that there are two processes, with different susceptibilities to FV, by which antibody-producing cells may be formed. One, which occurs mainly during the early part of the primary response, is insensitive to viral action. The other,

which first becomes apparent after the 2nd day of the primary response and from the onset of the secondary response, is strongly inhibited by FV.

Various authors (e.g. Vischer and Stastny, 1967; Cohen, Jacobson and Thorbecke, 1966; Leduc, Coons and Connolly, 1955) have suggested that a second contact with antigen may be a necessary part of the primary response. This 'secondary' part of the primary response, together with the secondary response proper, both perhaps involving the conversion of a primed progenitor cell to an antibody producing cell (Makinodan and Albright, 1963; Sercarz and Coons, 1962) could be inhibited by virus, while the first part of the primary response was not affected. Gowans and his colleagues (McGregor and Gowans, 1963; Ford and Gowans, 1967) have shown that circulating small lymphocytes are necessary for the primary response, while most of the cells responsible for the secondary response do not circulate. It may be that the former are the virus resistant and the latter the virus susceptible cells.

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