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PASSIVE TRANSFER OF ACQUIRED RESISTANCE IN MICE TO GROUP B ARBOVIRUSES BY SERUM PROTECTIVE FACTOR(S) (SPF) INDEPENDENT OF SERUM NEUTRALIZING ANTIBODY OR INTERFERON

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Thind, I. S. and W. H. Price (Schl. of Hygiene, The Johns Hopkins Univ., Baltimore, Md. 21205). Passive transfer of acquired resistance in mice to group B arboviruses by serum protective factor(s) (SPF) independent of serum neutralizing antibody or interferon. *Amer. J. Epid.*, 1968, *88*: 287–300.—Cross-protection between group B arboviruses cannot be directly related to the detectable serum neutralizing antibody or serum interferon levels at the time of challenge or subsequent to it. Cross-protection of similar magnitude is also found when cyclophosphamide, a potent immunosuppressant drug, is used both at the time of immunization and challenge. Such cross-protection has been related to another humoral factor called Serum Protective Factor (SPF). Some properties and the possible mechanism of action of SPF are outlined in this paper. SPF can be passively transferred and appears to play a dominant role in cross-protection. On primary infection, cyclophosphamide markedly increases the lethal titer of the parent strain of Langat virus, and also increases the titer of the vaccine strain, but to a lesser extent.

The neutralizing antibody and interferon levels in the serum of immunized mice are often at variance with the cross-protection observed against group B arboviruses (1). Furthermore, cross-protection between members of the group B arboviruses or group A arboviruses (2, 3) cannot be adequately predicted on the basis of antigenic cross-relationships in various serological tests. In the past, such disparity in results has not been completely explained although an anamnestic type of antibody response to the heterologous challenge virus has been one of the hypotheses put forward by Casals (2) and Hearn (3). However, both of these investigators also proposed an *altered host reaction* to account for their results.

Previous data from this laboratory have shown that the anamnestic neutralizing antibody response to the challenge virus is probably not the explanation of cross-protection (1). Further data also showed that blood from mice, immunized with Langat virus and treated with cyclophosphamide (Cytoxan), which contained no detectable serum neutralizing antibodies or interferon, on passive transfer was able to protect normal mice against a challenge

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of Powassan virus but not Japanese encephalitis virus. The specificity of this protection mechanism against particular challenge viruses was emphasized (1).

The results from previous experiments (1) and the results reported in this paper suggest that another serum factor(s), different from neutralizing antibody or interferon, plays the dominant role in resistance in cross-protection experiments. We have named this factor serum protective factor (SPF). The results reported in this paper illustrate the role of SPF in cross-protection and outline some of its properties.

Materials and methods

In general the methods of these experiments follow those described in detail in a previous paper (1). The experimental animals were six to seven-week-old (ICR) mice. The viruses used were an attenuated chick embryo strain of Langat (L) virus (TP21 M3E42 Egg ⅘) (4), Powassan (P) virus and Japanese encephalitis (JE) virus (1). All three viruses are members of Casals' group B arboviruses. The two former are members of the tick-borne virus complex. The latter is a mosquito-borne virus. The Langat and Powassan viruses are more closely related immunologically to each other than to JE virus. However, Powassan virus is the most distinct member of the tick-borne virus complex (5).

In all experiments reported in this paper, Langat virus was the primary immunizing agent. Mice were immunized with a single intraperitoneal (I.P.) injection of Langat virus; these are hereafter termed Langat-immunized mice. Other animals received 1.5 mg. of Cytoxan (cyclophosphamide, Mead, Johnson—a dose of about 50 mgm. per Kilo), an immunosuppressive agent, 24 hours before and 24 hours after Langat infection (1); these are called Cytoxan-treated, Langat-immunized mice. Both groups were bled 3 weeks later and the sera called Langat serum and Cytoxan-Langat serum, respectively. Other mice, after the bleeding, were challenged intraperitoneally with either P virus or JE virus. These mice were also treated with Cytoxan 24 hours before and 24 hours after the challenge inoculum (1). Some of these mice were bled by cardiac puncture 4 days after the challenge inoculum. These sera were called Cytoxan-Langat-Powassan serum and Cytoxan-Langat-JE serum, respectively. The rest of the mice were observed for mortality and calculation of per cent survival. Normal mice were inoculated intraperitoneally with 0.3 ml of 10 per cent normal chick embryo suspension. Some of these mice were treated with Cytoxan 24 hours before and 24 hours after the injection of chick embryo suspension. Such mice are called Control mice and Cytoxan-treated Control mice, respectively. Both groups of mice were bled 3 weeks later and the sera were called Control serum and Cytoxan-Control serum. Other Cytoxan-treated, Control mice were challenged intraperitoneally with either P virus or JE virus. These mice were also treated with Cytoxan 24 hours before and 24 hours after the inoculation of challenge virus. Some of these mice were bled 4 days later and the sera called Cytoxan-Control-Powassan serum and Cytoxan-Control-JE serum. The rest of these mice were observed for mortality to the challenge inoculum.

All challenge inocula of JE virus and Powassan virus used in this study refer to the age-specific intraperitoneal lethal doses for mice. Unless otherwise stated 10 to 15 ICR mice, 6 to 7 weeks old,

TABLE 1

The effect of neutralizing antibody level to Powassan virus acquired by passive transfer of serum on protection against Powassan virus challenge

Serum	Dilution of serum used	NI* of recipient mouse sera against P virus at time of challenge with P virus	Per cent survival against 500,000 challenge doses of P virus†
Control mouse serum	Undiluted	0	0
Langat mouse serum	Undiluted	0	70–90
Langat mouse hyperimmune serum	1:100	50	0
Powassan mouse hyperimmune serum	Undiluted	1,000	100
	1:10	630	90
	1:100	63	40
	1:1,000	25	0

* NI = Neutralization index of the pooled sera from 3 mice was determined by the mouse intraperitoneal neutralization test.

† = All observations are based on ten mice each except in case of Langat mouse serum where a total of 30 mice were used in two experiments.

were used in cross-challenge experiments, or to test the protective capacity of each serum specimen on passive transfer against each viral dilution. Fifty per cent endpoints were determined by the Reed and Muench method (6). Neutralization indices were determined by the mouse intraperitoneal neutralization test, the details of which have been furnished in a previous paper (1). Interferon activity was determined as described previously (1). Baby hamster kidney tissue cultures (BHK 21) were grown according to Karabatsos and Buckley (7).

RESULTS

Dissociation between protection and neutralizing antibody. Control mice show little resistance to intraperitoneal injection of P virus and the serum of Control mice (Control serum) is unable to neutralize this virus *in vitro* or confer protection[2] when given to normal mice 24 hours before challenge with P virus (table 1).

As reported earlier, the immunization of mice with Langat virus conferred protection against 500,000 intraperitoneal lethal doses of P virus (1). This *same* degree of protection was found when the serum of Langat-immunized mice (Langat serum) was inoculated 24 hours before challenge into normal mice (Table 1). Thus, the protective capacity is transferred with the serum. Undiluted unheated Langat serum has a neutralizing index of 32–50 against P virus. Passively immunized normal mice, which survive challenge with 500,000 LD_{50} of P virus, have no demonstrable serum neutralizing capacity against P virus at the time of challenge (Table 1). Thus, either a low undetectable level of neutralizing antibody plays a protective role in survival, or some other factor is responsible. The latter hypothesis is supported by the results of passive transfer experiments with Langat mouse hyperimmune serum and Powassan mouse hyperimmune serum (Table 1). When diluted hy-

[2] 0.4 ml serum of control mice was given as a single intraperitoneal injection per mouse 24 hours before the challenge inoculum.

TABLE 2

Protection against Powassan virus challenge in actively immunized mice or mice given passive transfer of serum

Serum treatment or immunized mice	Per cent survival against I.P. Powassan virus challenges*	
	320,000–500,000 LD_{50}	32–50 LD_{50}
Control mice	0	0–10
Control serum†	0	0–10
Normal serum + 1.5 logs P virus† ‡	0	0
Normal serum + 5.5 logs P virus† §	0	0–10
Langat-immunized mice	70–90	80–90
Cytoxan-treated, Langat-immunized mice	85–100	90–100
Cytoxan-Langat serum†	0	40–80
Cytoxan-Langat-Powassan serum† ‡	80–100	90–100
Cytoxan-Control-Powassan serum† §	0	0–10

* The figures represent the accumulated data of 3 separate experiments involving 30–35 mice per each specimen tested.

† 0.4 ml of serum given I.P. 24 and 3 hours before and 24 hours after the challenge inoculum. All sera were inactivated with Beta-propiolactone (BPL) (0.2% final concentration for 3 hours at room temperature) and then dialyzed against 0.1 M phosphate buffered saline pH 7.2 for 18 hours at 4 C. There was no detectable virus in any specimen after BPL inactivation.

‡ Cytoxan-Langat-Powassan serum had 1.5 logs of P virus per 0.02 ml before BPL treatment and no detectable virus after treatment. As a control for this specimen normal mouse serum was seeded with 1.5 logs of P virus (final concentration per 0.02 ml) and then inactivated with BPL.

§ Cytoxan-Control-Powassan serum had approximately 5.0 logs of P virus per 0.02 ml before BPL treatment. After treatment it had no detectable virus. As a control for this specimen normal serum was seeded with 5.5 logs of P virus (final concentration per 0.02 ml) and then inactivated with BPL.

perimmune sera are used for passive transfer, the subsequent protection to P virus is not related to the level of serum neutralizing antibody present at the time of challenge. However, if there is sufficient level of serum neutralizing antibody present at time of challenge, it does provide protection to challenge with P virus.

The discrepancy between antibody level and protection of passively immunized mice strongly suggest that undiluted unheated serum from Langat-immunized mice contains a factor(s) (SPF), other than neutralizing antibody, which conveys protection against P virus when injected in normal mice.

SPF and protection on passive transfer of serum. If antibodies capable of neutralizing virus *in vitro* should happen to coexist in the same animal or serum together with some other serum protective factor, it would be difficult to study them separately. In order to study the role of SPF, independent of neutralizing antibody, the immunosuppressant drug Cytoxan was employed (1). It has been shown by Santos that cells engaged in the primary response have identical sensitivity to immunosuppression by Cytoxan as do cells engaged in a secondary response (8).

Cytoxan-treated, Langat-immunized mice had about the same degree of protection against P virus as did Langat-immunized mice (table 2). However, the Cytoxan-Langat serum provided no protection against 500,000 LD_{50} of P virus, and only partial protection against 32–50 LD_{50} (table 2), in contrast to the protection against the higher level of challenge when serum from Langat-immunized mice (Langat serum) was used (table 1).

The degree of protection was depend-

ent on the dose of serum and the size of challenge inoculum. When 0.4 ml of Cytoxan-Langat serum was administered intraperitoneally 48, 24 and 3 hours before and 24 and 48 hours after challenge with 320 LD_{50} of P virus, 90 per cent protection was observed; a single dose of serum administered 48 or 3 hours before the challenge provided only 27 and 50 per cent protection, respectively.

It should be emphasized that Cytoxan-Langat serum had no demonstrable neutralizing antibody or interferon. Thus, the low level of protection must be due to a serum protective factor produced during treatment with the immunosuppressant drug Cytoxan. Earlier in this paper evidence was presented that the production of SPF could also occur in the sera of Langat-immunized mice *not treated* with Cytoxan.

Anamnestic SPF response and increased protection on passive transfer of serum. Cytoxan-treated, Langat-immunized mice show very high protection to P virus challenge in contrast to the sera from such mice which convey little protection against P virus when injected in normal mice. Further experiments were done to resolve this discrepancy. Cytoxan-treated, Langat-immunized mice were administered further Cytoxan along with the Powassan virus challenge (1) and the serum was collected 4 days later (Cytoxan-Langat-Powassan serum). This serum showed no demonstrable neutralizing antibody or interferon, yet when given to normal mice 24 and 3 hours before and 24 hours after challenge with P virus, it protected them as fully as the degree of protection found in Cytoxan-treated Langat-immunized mice (table 2). Evidently there is a large increase in SPF after administration of the second virus to a previously sensitized mouse. This might account for the ability of Cytoxan-treated, Langat-immunized mice to be protected against challenges with very large doses of P virus.

Specificity of anamnestic SPF response to the immunizing and the challenge viruses. Cytoxan-treated, Langat-immunized mice were treated with Cytoxan and challenged with JE virus. Serum collected from these mice 4 days after the JE challenge (Cytoxan-Langat-JE serum), when administered to normal mice, had no greater protective effect against JE virus challenge than did the Cytoxan-Langat serum (table 3). However, the JE virus did add to the resistance against Powassan virus, although the degree of protection was of a lower magnitude than that afforded by Cytoxan-Langat-Powassan serum. Cytoxan Langat-JE serum protected 30 per cent of the normal mice against 4,000,000 lethal doses of P virus, while the Cytoxan-Langat serum showed no protection against such a challenge dose (table 3). It is not understood why subsequent challenge with JE virus did not enhance the protective capacity of the serum against this virus.

SPF and in vivo *interferon production.* In order to determine whether the administration of the protective serum induces *in vivo* production of interferon in the recipient mouse, two inoculations of Cytoxan-Langat serum or Cytoxan-Control serum were administered to normal mice intraperitoneally 18 hours apart. Under these conditions protection against P virus challenge was demonstrable in normal mice given passive transfer of Cytoxan-Langat serum. Serum specimens were taken from the recipient mice 6, 12 and 24 hours

TABLE 3
Protection against P virus or JE virus challenge in mice given passive transfer of serum

Serum*	Per cent survival against challenge virus‡		
	Challenge doses of P virus		Challenge dose of JE virus
	4,000,000	400 LD_{50}	120 LD_{50}
Cytoxan-Control serum	0	20	31
Cytoxan-Control-JE serum	0	10	37
Cytoxan-Langat serum	0	30	33
Cytoxan-Langat-JE serum†	30	50	30
Cytoxan-Langat-Powassan serum†	70	87	Not done

* 0.4 ml of serum was given intraperitoneally 24 and 3 hours before and 24 hours after the challenge inoculum.

All sera were inactivated with Beta-propiolactone (BPL) (0.2% final concentration for 3 hours at room temperature) and then dialyzed against 0.1 M phosphate buffered saline pH 7.2 for 18 hours at 4 C. There was no detectable virus in any specimen after BPL inactivation.

† These sera had about 2.5–4.0 logs of suckling mouse I.C. LD_{50} of virus per 0.02 ml before BPL inactivation.

‡ 10–15 mice were used to determine per cent survival against each challenge dose of virus.

after the last inoculation and were tested for interferon activity by the methods previously described (1). These serum specimens showed no detectable interferon-like activity, although low concentrations of mouse serum interferon[8] used as a control in this experiment were easily detectable.

SPF and protection of baby hamster kidney (BHK 21) tissue culture cells. Cytoxan-Langat serum with no demonstrable neutralizing antibody or interferon was used in these experiments. The results are shown in table 4. BHK 21 cells were treated with a 10 per cent final concentration of unheated serum for 6 or 24 hours and then challenged with serial 10-fold dilutions of either P virus or JE virus. If the serum was left in the tubes throughout the infectious process, protection based on the absence of cytopathology was detected against 100 to 320 $TCID_{50}$ of P virus. Under similar conditions BHK 21 cells in the presence of normal mouse serum showed cytopathology. If the serum was washed off before the addition of the challenge virus inoculum, this protection was lost. This serum failed to protect tissue cultures against even the smallest amount of JE virus.

SPF and effect on virus in vitro. Cytoxan-Langat serum has no demonstrable neutralizing capacity in the mouse intraperitoneal neutralization test. Further evidence that this serum had no direct effect on virus was obtained as follows: 2.0 ml of undiluted serum was mixed with equal volumes of 10^{-6} or 10^{-7} dilutions of stock P virus and the mixture incubated for 2 hours at 37 C. It was then centrifuged at 40,000 rpm (144,700 × G) using a ⅘40 rotor in a Spinco Centrifuge for 2 hours. The supernatant fluid was poured off and the tube drained on a piece of filter paper. The sediment was resuspended and titrated for residual viral activity by ten-fold serial dilutions in BHK 21 cells. Table 5 shows that within the limits of experimental error the amount of virus recovered with Cytoxan-Langat serum was equal to that recovered with Cytoxan-Control serum. Data not shown in table 5 illustrated that the same amount of virus was recovered after *in vitro* treatment of P virus with Control serum and Cytoxan-Control serum. Langat serum (prepared by one inocula-

[8] The mouse serum interferon was kindly supplied by Dr. G. A. Cole.

TABLE 4

Titration endpoints of Powassan virus or JE virus on challenge of BHK 21 cells pretreated with serum

Experiment number	Serum	Serum left in or washed	Interval in hours between treatment and challenge	Titration end point ($TCID_{50}$) against challenge virus	
				Powassan virus	JE virus
1	Cytoxan-Control serum	Left in	24	7.5 or more*	9.0
		Washed	24	7.5 or more	9.5 or more
		Left in	6	7.5 or more	9.0
		Washed	6	7.5 or more	9.5 or more
	Cytoxan-Langat serum	Left in	24	6.0	9.5 or more
		Washed	24	7.5 or more	9.5 or more
		Left in	6	5.0	9.0
		Washed	6	7.5 or more	9.5 or more
2	Cytoxan-Control serum	Left in	6	8.0	ND
		Washed	6	7.5	ND
	Cytoxan-Langat serum	Left in	6	5.5	ND
		Washed	6	7.5	ND

* In viral titrations where end points were not reached, there was no difference in relation to time of appearance and degree of cytopathogenic effects observed in tubes pretreated with either Cytoxan-Control serum or Cytoxan-Langat serum. Four tubes were used to test each viral dilution.

TABLE 5

Residual infectivity after in-vitro serum treament of Powassan virus detected by viral titrations using BHK 21 cells

Experiment number	Serum	N.I.* to P virus	Dilution of P virus treated with serum	Residual infectivity ($TCID_{50}$)
1	Cytoxan-Control serum	0	10^{-6}	320
			10^{-7}	100
	Cytoxan-Langat serum	0	10^{-6}	100
			10^{-7}	100
2	Cytoxan-Control serum	0	10^{-6}	100
			10^{-7}	10
	Cytoxan-Langat serum	0	10^{-6}	320
			10^{-7}	10
	Langat serum	32	10^{-7}	1.0
	Monkey-Langat-Powassan serum	10,000	10^{-4}	320
			10^{-7}	<1.0

* NI = Neutralization index determined by mouse intraperitoneal neutralization test.

tion of Langat virus followed by bleeding 3 weeks later) having a mouse neutralization index of 32 against Powassan virus, when mixed with P virus, reduced its infectivity from 10 to 1 $TCID_{50}$ (table 5). The serum of monkeys, hyperimmunized successively with Langat and Powassan viruses, having a neutralization index of 10,000 against P virus when mixed with 10,000 $TCID_{50}$ of that virus, reduced its infectivity to 320.

Data in table 5 would argue against the possibilities that if the serum neutralizing antibody was present it would not combine with virus or that the neutralizing antibody-virus complex dissociated under the experimental conditions. Such observations suggest that SPF has no direct effect on the infectivity of virus *in vitro*.

Physical and chemical properties of SPF

Effect of heat inactivation on SPF. Undiluted unheated Langat serum when

TABLE 6

*Titration endpoints of Powassan virus on challenge of BHK 21 cells pretreated with mercaptoethanol-treated serum or other sera**

Serum	Serum treatment	Titration endpoint ($TCID_{50}$)
Cytoxan-Control serum	Mercaptoethanol-dialyzed†	8.5
	Phosphate buffered saline-dialyzed‡	9.0
	Phosphate buffered saline§	9.0
Cytoxan-Langat serum	Mercaptoethanol-dialyzed†	7.0
	Phosphate buffered saline-dialyzed‡	7.0
	Phosphate buffered saline§	6.5

* BHK 21 cells were treated with the respective serum 6 hours before challenge with P virus and the serum was left in during the entire period of the infectious process.

† Serum was mixed with an equal volume of mercaptoethanol, kept at 4 C for 18 hours and then dialyzed against 0.1 M phosphate buffer pH 7.2 containing 0.85% saline for 18 hours at 4 C. This serum was used for treatment of BHK 21 cells.

‡ Serum was mixed with an equal volume of phosphate buffered saline, kept at 4 C for 18 hours and then dialyzed against 0.1 M phosphate buffer saline pH 7.2 for 18 hours at 4 C before using for treatment of BHK 21 cells.

§ Serum was mixed with an equal volume of 0.1 M phosphate buffer saline pH 7.2, kept at 4 C for 36 hours and then used for treatment of BHK 21 cells.

injected into normal mice conveys 70–90 per cent protection against 500,000 lethal doses of P virus (table 1). The treatment of this serum at 56 C for 30 minutes led to an 80–90 per cent reduction in its capacity to confer protection. Similar data were also obtained for heat inactivated Cytoxan-Langat serum with BHK 21 cells. The protective capacity was not restored by addition to the heated serum of fresh homologous normal mouse serum in a final concentration of 20 per cent, indicating that the heating had affected the SPF itself and not heat-sensitive accessory factor present in normal serum. The heat sensitivity of SPF was reported previously (1). Control experiments showed that treatment at 56 C for 30 minutes did not reduce the level of neutralizing antibody as detected by *in vitro* neutralization tests in suckling mice.

Effect of acid treatment on SPF. Cytoxan-Langat serum and Cytoxan-Langat-Powassan serum can passively protect normal mice against P virus challenge (table 2). The pH of such sera was lowered to 2.0–2.5 for 3 hours at 4 C, then readjusted to pH 7.2–7.4 and injected into normal mice which were later challenged with 50 or 500,000 lethal doses of P virus. Such treatment led to complete removal of the protective effect. Interferon was stable under such conditions while serum neutralizing antibody was destroyed.

Effect of 2-mercaptoethanol on SPF. Cytoxan-Langat serum and Cytoxan-Control serum were treated with 2-mercaptoethanol for the reduction of macroglobulin, according to the method of Svehag (9). Suitable controls were included. BHK 21 cells were treated with 10 per cent final concentrations of these treated sera, 6 hours before challenge with serial 10-fold dilutions of stock Powassan virus. The sera were left in the tubes during the entire period of infectious process. Table 6 shows that such treatment had no effect on the protective value of Cytoxan-Langat serum against Powassan virus in tissue culture. Langat serum collected 10 days after the inoculation of the virus and having 19S antibody showed an eight-fold de-

crease in serum neutralizing antibody titer when treated with 2-mercaptoethanol under the same conditions.

Effect of dialysis on SPF, neutralizing antibody and interferon. Preliminary studies have shown that SPF is non-dialyzable through cellophane tubing with an average pore diameter of 48 Angstrom units. Cytoxan-Langat serum and Cytoxan-Langat-Powassan serum was dialyzed against 0.1 M phosphate buffer, pH 7.2, containing 0.85 per cent saline for 24 hours at 4 C. Cytoxan-Control serum and Cytoxan-Control-P serum were included as controls. Approximately 85 per cent of the protective activity was recovered in the non-dialyzable portion of the Cytoxan-Langat and Cytoxan-Langat-Powassan sera when tested in the mouse protection system.

Further studies were done by dialyzing the above mentioned 4 serum specimens against large volumes of distilled water for 48 hours at 4 C. The specimens were centrifuged, the supernatant poured off and the precipitate was then redissolved and restored to the original volume in 0.1 M phosphate buffer, pH 7.2, containing 0.85 per cent saline. Mice were given 0.4 ml intraperitoneally of either the supernatant fluid, the redissolved precipitate, or 0.8 ml of combined supernatant fluid and redissolved precipitate 24 and 3 hours before and 24 hours after the challenge inoculum of P virus. There was complete loss of protective activity of Cytoxan-Langat and Cytoxan-Langat-Powassan sera, when the supernatant fluid, redissolved precipitate, or the combined two fractions were tested under the above conditions (table 7). No protective activity was detected with any fractions of the control serum specimens as well. Only data with the combined two fractions of Cytoxan-Langat-Powassan serum and Cytoxan-Control serum are included in table 7.

Under similar conditions of water dialysis the protective capability of serum neutralizing antibody, when tested on passive transfer in the mouse protection system, was not lost (table 7). Similarly, the interferon activity of mouse serum interferon was not destroyed (table 8).

Effect of Cytoxan on Langat infection in mice. Data presented in an earlier paper (1) and in this paper show that administration of Cytoxan, both at the time of primary immunization and at challenge, had no effect on the cross-protection observed when Langat-immunized mice were challenged with Powassan virus. However, the administration of Cytoxan did affect the outcome of primary Langat vaccine infection in mice as shown in table 9. Three weeks after the primary infection there was no detectable neutralizing antibody in serum, liver, or spleen to Langat virus in the surviving Cytoxan-treated mice (10).

Cytoxan also increases the virulence of the parent strain of Langat virus. The data in table 10 show that the virulence of the parent strain of Langat virus is increased far more than the vaccine strain of Langat virus. Thus, approximately equivalent concentrations of both viruses are needed to give a 50 per cent endpoint on intraperitoneal inoculation in the absence of Cytoxan, while with Cytoxan 1.3 logs of virus are needed for the parent strain and about 5.0 logs of virus for the vaccine strain.

Discussion

In this paper we report some properties of SPF produced in mice by Langat virus infections with or without Cytoxan treatment. SPF, probably not

TABLE 7

Protection against Powassan virus challenge in mice given passive transfer of untreated or water-dialyzed serum

Serum	Active component in sera before treatment	Serum treatment‡	Serum dilution§	Per cent survival against I.P. challenge of P virus**	
				6,300,000	630
Cytoxan-Control serum*	None	None	Undiluted	0	10
		Water dialysis	Undiluted	0	0
Cytoxan-Langat-Powassan serum*	SPF	None	Undiluted	72	88
		Water dialysis	Undiluted	0	20
Monkey Langat-Powassan hyperimmune serum†	Neutralizing antibody	None	Undiluted	100	ND
			1:10	20	60
			1:100	10	10
		Water dialysis	Undiluted	100	ND
			1:10	10	50
			1:100	0	20
Mouse Powassan hyperimmune serum†	Neutralizing antibody	None	Undiluted	70	ND
			1:10	60	87
			1:100	0	60
		Water dialysis	Undiluted	100	ND
			1:10	60	80
			1:100	0	30

* These sera were *not* heat-inactivated and 0.4 ml volumes were given intraperitoneally to normal mice 24 and 3 hours before and 24 hours after the challenge inoculum.

† These sera were heat-inactivated at 56 C for 30 minutes and given intraperitoneally to normal mice 24 hours before the challenge inoculum in 0.4 ml volume.

‡ Some sera were dialyzed against large volumes of distilled water for 48 hours at 4 C. The precipitate was redissolved to original serum volume in 0.1 M phosphate buffer, pH 7.2, containing 0.85% saline and combined with the supernatant fluid. The combined fractions in 0.8 ml volumes, which represented 0.4 ml of the original serum specimen, was used in passive transfer to each normal mouse.

Sera not dialysed as above were kept at 4 C for 48 hours and then 0.4 ml was used in passive transfer to each normal mouse.

§ All dilutions were made in a final concentration of 20 per cent fresh homologous serum.

** Per cent survivals were calculated based on mortality ratios in 10–15 mice per challenge dose.

of a macroglobulin nature, is a heat and acid labile non-dialyzable factor. After dialysis against water, the protective capacity of SPF is lost, while it is not lost on dialysis against 0.1 M phosphate buffer, pH 7.2, containing 0.85 per cent saline. SPF has no direct detectable effect on P virus. However, SPF, on passive transfer, protects both mice and BHK 21 tissue culture cells against Powassan virus but not against JE virus challenge. This protective effect of SPF is probably mediated through cells, either of tissue culture or of the recipient host. This suggests that SPF, like interferon, is a factor that can confer cellular resistance, but differs from interferon in its virus specificity.

The data indicate that SPF must be present before and during the infectious process. Whether SPF affects the viral adsorption, penetration, or some stage in viral synthesis is not known at the present time.

It should be emphasized that Cytoxan-treated, Langat-immunized mice and Langat-immunized mice have similar protective ability against P virus challenge in spite of the fact that there is no detectable serum neutralizing antibody produced in the former mice as contrasted to a neutralizing index of 5,000–10,000 against P virus in the latter mice (1). However, there is a wide disparity in the amounts of protection observed in Cytoxan-treated, Langat-immunized mice and normal mice given Cytoxan-Langat serum (table 2). Experimental data presented in this paper indicate that the reason for this difference is the fact that the amount of SPF increases early and to higher titers after challenge of Cytoxan-treated, Langat-immunized mice with Powassan virus. Such an interpretation is substantiated by the protective ability of Cytoxan-Langat-Powassan serum on passive transfer.

In order for the anamnestic SPF response to be produced by the challenge virus *against itself* there must be some antigenic relationship between the immunizing virus and the challenge

TABLE 8

Effect of water dialysis on activity of mouse serum interferon

Serum	Serum treatment*	Titer against 50 $TCID_{50}$
Mouse serum interferon	None	1:128
Mouse serum interferon	Dialyzed against water	1:128

* Serum was dialyzed against large volumes of distilled water for 48 hours at 4 C. The precipitate was redissolved to the original serum volume in 0.1 M phosphate buffer, pH 7.2, containing 0.85% saline and then combined with the supernatant fluid. The combined fractions were used for testing interferon activity. The serum specimen not dialyzed as above was kept at 4 C for 48 hours and then tested for interferon activity. Four tissue culture tubes were used per dilution of serum.

TABLE 9

The effect of Cytoxan on the virulence of the vaccine strain of Langat virus in mice

Treatment	Number of mice	Per cent mortality
0.3 ml 10% normal chick embryo (NCE) I.P.	210	2.4
Langat vaccine I.P.*	258	5.8
1.5 mg. Cytoxan† followed by NCE followed 24 hours later by 1.5 mg. Cytoxan	470	10.4
1.5 mg. Cytoxan† followed by Langat vaccine I.P.* followed 24 hours later by 1.5 mg. of Cytoxan	2,750	59.1

* Dose equivalent to $10^{5.5}$ I.C. LD_{50} in three-week-old mice.

† Dose approximately 50 mg. per kilogram body weight.

TABLE 10

The effect of Cytoxan on the intraperitoneal virulence of the parent and the vaccine strain of Langat virus

Virus	I.P. titer*		I.C. titer‡
	Treatment		Treatment
	Cytoxan†	None	None
Parent	7.7	3.1	9.0
Vaccine	1.0	<1.0	6.0

* Reciprocal of the logarithm (base 10) in 6-week-old mice per 0.3 ml.

† Cytoxan treatment was the same as shown in table 9.

‡ Reciprocal of the logarithm (base 10) in 3-week-old mice inoculated intracerebrally. Values are corrected for a volume of 0.3 ml.

TABLE 11

Comparative properties of serum protective factor, serum interferon, and serum neutralizing antibody

Property	SPF	Interferon	Neutralizing antibody
Specific for virus	+	−	+
Produced under treatment with Cytoxan	+	+	−
Direct action on viruses	−	−	+
Heat stability (56 C for 30 minutes)	±	+	+
Stable to acid	−	+	−
Non-dialyzable	+	+	+
Inactivation by water dialysis	+	−	−
Protection of mice by passive transfer	+	+	+
Protection by treatment of tissue culture cells relative to infection	Before & during infectious process	Before or after start of infectious process	No direct action on cells
Time of appearance in infectious process	Second	First	Third
Persistence	Long lasting	Short lived	Long lasting
Effect of anamnestic response to challenge virus on viremia	Lowered	No effect or ?	No effect or lowered
Anamnestic response with cross-challenge protection	+	+	+
Anamnestic response without cross-challenge protection	−	+	+

virus. Furthermore, the anamnestic SPF response may follow the "Doctrine of Original Antigenic Sin" (11). Cytoxan-treated, Langat-immunized mice challenged with JE virus and given Cytoxan produce increased amounts of SPF only against P virus and not against JE virus. The reverse order could not be studied since mice given JE virus and treated with Cytoxan or P virus and administered Cytoxan as the immunizing viruses would not survive these primary infections. This relationship between the immunizing virus and the challenge virus in the production of SPF must be other than that which determines the neutralizing antibody response on cross-challenge, as an anamnestic neutralizing antibody response is observed against JE virus when it is given to Langat sensitized mice (1).

The protective sera which convey resistance when injected into normal mice do not induce the *in vivo* production of interferon-like substances in the recipient host. Furthermore, Cytoxan-Langat and Cytoxan-Langat-Powassan sera on passive transfer to normal mice confer resistance to Powassan virus only and not JE virus. This resistance on passive transfer of sera had the *same specificity* that was found in the actively immunized mice. Thus, the sera retain the original specificity of acquired host resistance.

The protective sera have no detectable neutralizing antibody or interferon-like activity, and yet confer protection to normal mice on passive transfer. This strongly suggests that some factor(s) other than serum neutralizing antibody or serum interferon must be responsible for the full expression of acquired resistance by passive transfer of serum.

There is not yet enough data available to evaluate the relative roles of SPF,

interferon and neutralizing antibody definitively in viral resistance. However, in the cross-protection systems that have been studied in this laboratory it would appear that SPF plays a more dominant role than interferon or neutralizing antibody in resistance. This is supported by the fact that an anamnestic response of SPF *against the challenge virus* results in resistance to the challenge virus. In contrast, systems have been studied in which there is early formation of large amounts of interferon or neutralizing antibody but no protection (1) and no production of SPF against the challenge virus. Furthermore, serum neutralizing antibody can be inhibited by the use of Cytoxan with no effect on cross-protection (1).

Data presented in this paper have shown that Cytoxan can increase the virulence of the avirulent Langat isolate, resulting in the death of a greater number of mice. In the absence of Cytoxan, similar concentrations of the parent and vaccine viruses give a 50 per cent endpoint on intraperitoneal inoculation. In the presence of Cytoxan, however, far less parent virus than vaccine virus is needed to give a 50 per cent endpoint. These results suggest that under these conditions one of the factors that results in a change in virulence on Cytoxan treatment is dependent upon the virulent potentiality of the virus.

A brief summary of the properties of SPF, serum interferon and serum neutralizing antibody is included in table 11.

Addendum

Additional properties of SPF

Since the above paper was submitted for publication, work by Winston H. Price, Inderjit S. Thind, Harvey Harrison, John Molenda, Robert Prendergast and John Myers has revealed several new properties of SPF.

The biological activity of SPF in the Cytoxan-Langat-Powassan serum is precipitated in the 20–50 per cent saturated ammonium sulfate fraction. When Cytoxan-Langat-Powassan serum is fractionated on Sephadex G-100 none of the SPF activity appears in the fractions which should contain all of the 19S antibodies of serum. All of the biologic activity of SPF is found in the fraction usually associated with 7S antibody. However, this serum does not neutralize the virus *in vitro*.

SPF has been purified on polyacrylamide gels using disc electrophoresis.

SPF is produced on primary immunization with the attenuated strain of Langat virus 3 days before the detectable appearance of serum neutralizing antibody and appears in the serum of mice about 2 days after the virus is given. On primary infection with Langat virus SPF is greatly inhibited by treatment of mice with Cytoxan.

The viral titers in the brains of mice given Cytoxan treatment and the Langat attenuated virus strain are much higher and persist much longer than the viral titers found in the brains of mice given only the attenuated strain of Langat virus. This effect may be related to the increased mortality due to Cytoxan treatment of Langat virus inoculated mice. Whatever protective mechanism(s) is inhibited by Cytoxan is associated with increased viral multiplication in the brains of the infected mice. It is of interest that if SPF is given 24 hours before or after 100 challenge doses of Langat virus to mice treated with Cytoxan, most of the mice are protected. Further data indicate that the suppression of serum neutralizing antibodies does not play a major role in the increased virulence of the attenuated strain of Langat virus in mice treated with Cytoxan at the time of immunization. Other data indicate that since high levels of serum interferon are produced in mice inoculated with the attenuated strain of Langat virus given Cytoxan treatment, it does not seem likely that interferon could account for the increased virulence of Langat virus under Cytoxan treatment.

Two inoculations of Beta-propriolactone inactivated Langat virus or West Nile virus protected mice against at least 10,000 intraperitoneal challenge doses of homologous live virus. However, when Cytoxan was given with inactivated vaccine inoculations and at the time of challenge no protection was detectable even against low challenge inocula of homo-

logous viruses. The treatment with Cytoxan was the same as that described in the above paper with liver virus vaccination. In the mice immunized with inactivated vaccines and treated with Cytoxan there was no secondary SPF response following the challenge inoculum as was observed with live virus vaccination reported in the above paper. These results indicate that protection due to inactivated vaccines is probably due to the production of serum neutralizing antibody and not to SPF in contrast to the cross-protection found with live virus vaccinations.

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