Table 2. Allele frequencies at 15 loci in three carangid species.

Locus	Allele	Species Dr(22)	Sl(6)	Sc(2)
α-Gpd	185	0.0	1.0	0.0
	154	0.0	0.0	1.0
	100	1.0	0.0	0.0
Ldh-1	124	1.0	0.0	0.0
	100	0.0	1.0	1.0
Ldh-2	100	1.0	1.0	1.0
Ldh-3	−60	1.0	0.0	0.0
	−100	0.0	1.0	1.0
Mdh-1	160	0.0	1.0	0.0
	100	1.0	0.0	1.0
Mdh-2	−100	1.0	1.0	1.0
Ck	150	1.0	0.0	0.0
	125	0.0	1.0	0.0
	100	0.0	0.0	1.0
Fm	120	0.0	1.0	0.0
	180	0.0	0.0	1.0
	100	1.0	0.0	0.0
Idh	135	0.0	1.0	0.0
	100	1.0	0.0	1.0
Me-1	100	1.0	0.0	0.0
	90	0.0	1.0	1.0
Me-2	100	1.0	0.0	0.0
	77	0.0	1.0	1.0
Sp-1	−40	0.318	0.0	0.0
	−80	0.682	0.0	0.0
	−100	0.0	1.0	1.0
Sp-2	−100	0.0	1.0	1.0
	−105	1.0	0.0	0.0
Hem-1	141	1.0	0.0	0.0
	100	0.0	1.0	1.0
Hem-2	100	0.0	1.0	1.0
	−75	1.0	0.0	0.0

Figures in parentheses show numbers of individuals examined. Dr, *Decapterus russelli*; Sl, *Selaroides leptolepis*; Se, *Selar crumenophthalmus*.

Table 3. Estimates of genetic distance (D) along with standard error between three carangid species. Values above the diagonal are estimates of D, those below are estimates of divergence time (in million years).

Species	1	2	3
Selaroides leptolepis	—	0.470 ± 0.258	2.066 ± 0.874
Selar crumenophthalmus	2.35 ± 1.29	—	1.373 ± 0.573
Decapterus russelli	10.33 ± 4.37	6.87 ± 2.86	—

have the advantage that they vary linearly with the phylogenetic divergence time[10].

To estimate the relationships among the three genera, a dendrogram was drawn based on the genetic distances (Figure 2). From this dendrogram, the three species were clearly divisible into two major groups at a distance of 1.72. One of the groups had *S. leptolepis* and *S. crumenopthalmus* while the other group consisted of *D. russelli*.

Biochemical diversity measured as genetic distance was reported among species in many organisms[14]. Nei[15] estimated that for local races of a species, D ranges from nearly 0 to 0.05; for subspecies $D = 0.02$ to 0.2. For full species, $D = 0.10$ to 2.0; sibling species, which are morphologically nearly identical, generally have D values two to three times smaller than the Ds between morphologically distinct species. D is usually greater than 1.0 for genera. In Carangidae, collected from Okinawa and Kochi, Japan, Kijima *et al.*[16], using 6 enzymes and 9 loci coding for them, indicated the average genetic distance as 2.20 between *D. russelli* and *S. leptolepis*; 1.64 between *D. russelli* and *S. crumenophthalmus* and 0.79 between *S. leptolepis* and *S. crumenophthalmus*. In sparid fish, the average genetic distance was reported as 0.002 between conspecific subpopulations, 0.115 between congeneric species, 0.842 between consubfamilial genera and 1.273 between subfamilies[17]. In Sciaenidae[5], the average genetic distance varied between 0.0017 and 0.039 for conspecific subpopulations, 0.092 between two species of Nibea and 1.212 between consubfamilial genera. Relationships between standard genetic distances observed for various taxonomic categories are about as expected. Higher categories are on the average more different than lower ones. The present estimates of genetic distance are more or less similar to that of Kijima *et al.*[16] and show that the structural gene difference between *Selaroides leptolepis* and *Selar crumenophthalmus* is quite small. In estimating genetic distance a large number of loci rather than a large number of individuals per locus should be used when the total number of genes to be examined is fixed[18]. Ideally, more than 50 loci should be used to obtain reliable estimate[18]. In practice, technical difficulties often limit the number of loci studied. In fact, less than 30 loci have been studied in most protein surveys. A large number of individuals must be examined if one wants to study the allele frequency distribution for each locus.

The rapidly expanding application of multilocus electrophoretic techniques to the analysis of systematic and evolutionary problems is transforming the taxonomic work. Building up of a systematic arrangement more consistent with the genetic relationships of organisms is expected as a major result of this new approach[19].

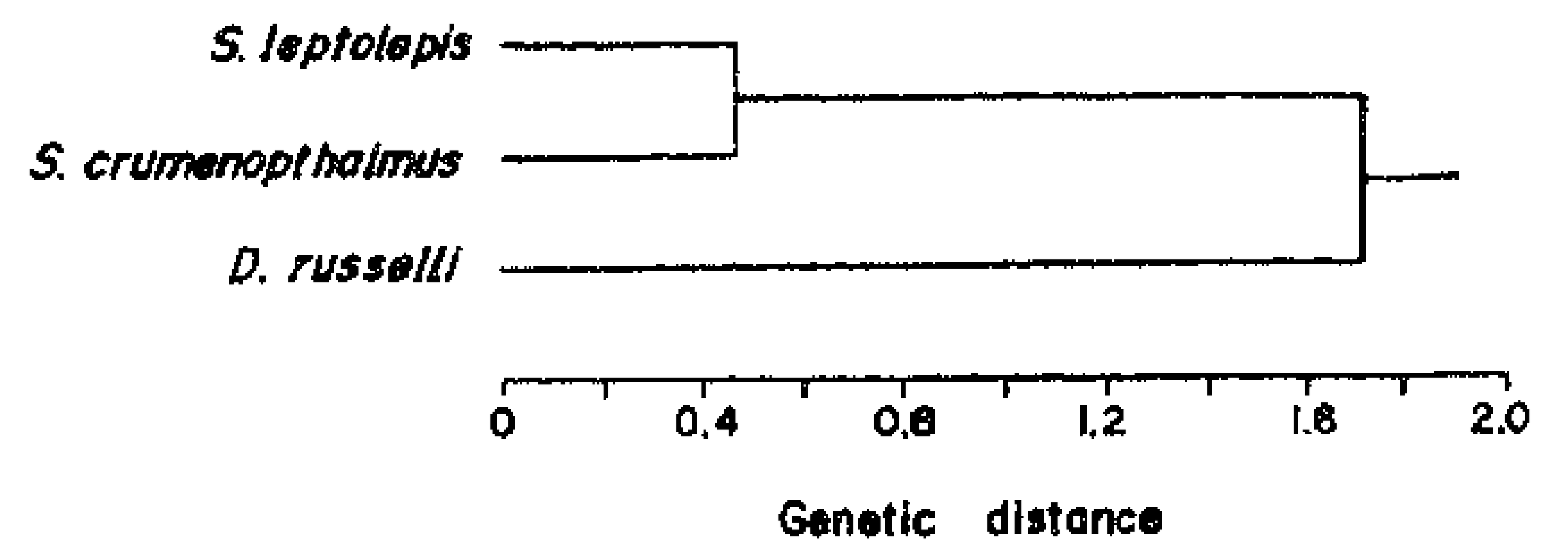

Figure 2. Dendrogram showing the relationship among the 3 carangid species based on values of genetic distance.

1. Hunter, R. and Markert, C., *Science*, 1957, **125**, 1294.

2. Allendorf, F. W. and Utter, F. M., in *Fish Physiology* (ed. Hoar, W. S. and Randall, D. J.), Academic Press, New York, 1979, vol. 8, p. 407.
3. Wilkins, N. P., *Aquaculture*, 1981, **22**, 209.
4. McAndrew, B. J. and Majumdar, K. C., *Aquaculture*, 1983, **30**, 249.
5. Menezes, M. R. and Taniguchi, N., *Jpn. J. Ichthylol.*, 1988, **35**, 40.
6. Hartl, D. L., *Principles of Population Genetics*, Sinauer Associates, Inc., Sunderland, Mass., 1980, p. 488.
7. Bal, D. V. and Rao, K. V., *Marine Fisheries*,, Tata McGraw Hill Publishing Company Limited, New Delhi, 1984, p. 470.
8. Taniguchi, N. and Numachi, K., *Bull. Jpn. Soc. Sci. Fish.*, 1978, **44**, 1351.
9. Shaw, C. R. and Prasad, R., *Biochem. Genet.*, 1970, **4**, 297.
10. Nozawa, K., Shotake, T., Kawamoto, Y. and Tanabe, Y., *Primates*, 1982, **23**, 432.
11. Nei, M., *Am. Nat.*, 1971, **105**, 385.
12. Nei, M., *Molecular Population Genetics and Evolution*, North Holland, Amsterdam, 1975.
13. Sneath, P. H. A. and Sokal, R. R., *Numerical Taxonomy*, W. H. Freeman and Company, San Francisco, 1973, p. 573.
14. Ayala, F. J., in *Protein Polymorphism: Adaptive and Taxonomic Significance* (eds. Oxford, G. S. and Rollinson, D.), Academic Press, London, 1983, p. 3.
15. Nei, M., in *Population genetics and ecology* (eds. Karlin, S. and Nevo, E.), Academic Press, New York, 1976, p. 723.
16. Kijima, A., Taniguchi, N. and Ochiai, A., in *Indo-Pacific Fish Biology: Proc. of the 2nd Int. Conf on Indo-Pacific Fishes*, Ichthylological Society of Japan, Tokyo, 1986, p. 840.
17. Taniguchi, N., Fujita, M. and Akazaki, M., in *Indo-Pacific Fish Biology: Proc. of the 2nd Int. Conf. on Indo-Pacific Fishes*, Ichthyological Society of Japan, Tokyo, 1986, p. 849.
18. Nei, M., *Genetics*, 1978, **89**, 583.
19. Bullini, L., in *Protein Polymorphism: Adaptive and Taxonomic Significance*, (eds. Oxford, G. S. and Rollinson, D.), Academic Press, London, 1983, p. 179.

ACKNOWLEDGEMENTS. I thank Dr A. H. Parulekar for going through the manuscript. This study was supported by a grant from the Department of Ocean Development, New Delhi.

10 December 1988; revised 19 October 1989

Effect of Japanese encephalitis virus-induced suppressor cells on Fc-receptor function of mouse macrophages

Shashi Rawat, Asha Mathur, R. Kulshreshtha and U. C. Chaturvedi

Postgraduate Department of Microbiology, K. G. Medical College, Lucknow 226 003, India

During Japanese encephalitis virus (JEV) infection Fc-receptor-mediated attachment of opsonized sheep erythrocytes (EA) by macrophages was significantly depressed. The capacity of macrophages to attach EA reached the lowest point on day 18 following intraperitoneal inoculation of JEV and then recovered gradually, but remained less than the controls. We have observed earlier that T lymphocytes of JEV infected mouse spleen produce a suppressor factor on the 18th day p.i. The present study shows that JEV-induced suppressor cells have a variable effect on the activity of macrophages. The second generation of the suppressor T cells (Ts_2) significantly depressed the Fc-receptor-mediated attachment of EA. Further simultaneous administration of JEV and suppressor factor (SF) resulted in early depression in macrophage Fc-receptor-mediated attachment of EA compared to JEV alone. Thus SF appears to protect the cells by inhibiting the entry of virus into macrophages.

MACROPHAGES play an important role in the immune response to an antigen. The various functions of macrophages are regulated by extracellular signals that operate via different types of receptors present on the surface of macrophages. Mouse macrophages have four types of receptors for the Fc portion of immunoglobulin and are controlled by two genes[1,2]. The extracellular signals can increase the Fc-receptor-mediated activity, as has been demonstrated with lymphokines[3] and gamma interferon[4], or decrease them as has been observed with prostaglandins and macroglobulin protease complexes[5].

We have earlier reported that JEV induces generation of suppressor cells for the humoral and cell-mediated immune responses during the 3rd week of infection which mediate suppression through suppressor factor[6,7]. The live macrophages are obligatory for the transmission of the suppressor signal[8]. In the present study we have studied the Fc-receptor-mediated function of macrophages in JEV-infected mice and the effect of suppressor cells/suppressor factor on them. The findings show that during JEV infection the Fc-receptor-mediated functions are depressed due to the generation of suppressor pathway.

Methods

Preparation of suppressor cells (Ts_1 population). Two-to-three months old inbred Swiss albino mice from the

colony maintained in the department were inoculated with 10^3 LD_{50} of JEV intraperitoneally. The virus, strain 78668A, was propagated in adult mouse brain[7], and used in the form of a 10% suspension (w/v). The inoculated mice were sacrificed on day 18 (time of appearance of suppressor activity[7]) and their spleens were collected aseptically. Single cell suspension was prepared in MEM solution and used as the first generation suppressor T cells[6].

Preparation of suppressor factor. The suppressor factor (SF) was prepared as described previously[10]. Briefly, the spleens collected on day 18 as described above were homogenized in phosphate-buffered saline (PBS). The homogenate was centrifuged at 100,000 *g* for 90 min in the cold, the supernatant was stored in aliquots at −70°C and used as SF. Normal mouse spleen homogenate similarly prepared was used in controls.

Preparation of second generation suppressor cells (Ts_2). Normal mouse spleen cells incubated with SF for 1 h at 37°C were washed thrice and cultured. After 24 h the cells were harvested and used as second generation suppressor T cells (Ts_2)[8].

Peritoneal macrophage culture. The peritoneal cavity of the mouse was lavaged with 4 ml heparinized MEM containing antibiotics and fetal calf serum. The peritoneal fluid was collected, cells were washed and 5×10^6 cells were layered on 20×20 mm coverslips placed in petri dishes. After incubation at 37°C for 2 h in an atmosphere of 5% CO_2 the coverslips were washed thoroughly with MEM to remove non-adherent cells. The glass adherent cells were considered macrophages on the basis of morphology and latex particle phagocytosis.

Sheep red blood cells rosettes. Opsonized sheep erythrocytes (EA) were prepared by the technique of Stuart *et al.*[11] Briefly, sheep erythrocytes (SRBC) were washed thrice with saline and treated with heat-inactivated rabbit anti-sheep erythrocytes (haemolysin) at 37°C for 30 min and then washed in PBS. One per cent suspension of coated red cells was prepared in PBS and used fresh. SRBC-treated similarly without coating with haemolysin were used as controls for background attachment of unopsonized RBC. The macrophage (Mϕ) culture sheet was incubated with freshly opsonized SRBC (100 EA/Mϕ) at 37°C for 1 h. The cell sheet was washed thoroughly and examined for the attachment of opsonized SRBC (EA). The number of EA attached per macrophage was counted in 100 or more cells on duplicate coverslips from each mouse. Mean values of 12 to 14 observations with standard deviation have been presented after deducting the background attachment of unopsonized RBC. The data were analysed using Student's *t* test.

Protocol. The effect of JEV infection, Ts_1, Ts_2 cells and suppressor factor on Fc-mediated attachment of opsonized SRBC by macrophages was studied. The mice were injected i.p. with JEV or SF and i.v. with Ts_1 or Ts_2 cells (1×10^8 cells/0.25 ml). The mice were sacrificed on alternate days from day 1 to day 26 p.i. Normal mice were included in each experiment as controls. The peritoneal macrophage monolayers on coverslips were prepared and assayed for rosetting of opsonized SRBC. The tests were done in triplicate macrophage monolayers and the mean value as obtained (±SD) from 5 to 7 mice in each group after deducting background binding of non-opsonized SRBC is presented.

Results

Effect of JEV infection on Fc-receptor-mediated attachment

Figure 1 shows Fc-receptor-mediated attachment of opsonized SRBC (EA) following i.p. inoculation of JEV at different periods. The mean per cent rosette-forming cells on different days in normal control mice were 75±4.4%. No significant day-to-day variation was seen in the attachment values of EA in controls while JEV infection diminished the rosette-forming capacity of macrophages and the values remained lower than the controls throughout the study. The lowest values were observed on day 18 (46±5%).

Effect of Ts_1 and Ts_2 cells on Fc-receptor-mediated attachment

Mice were injected with either Ts_1 or Ts_2 cells (1×10^8) intravenously. The peritoneal macrophages of recipient mice were collected on day 1 and then at 4-day intervals of the cell transfer and studied for Fc-receptor

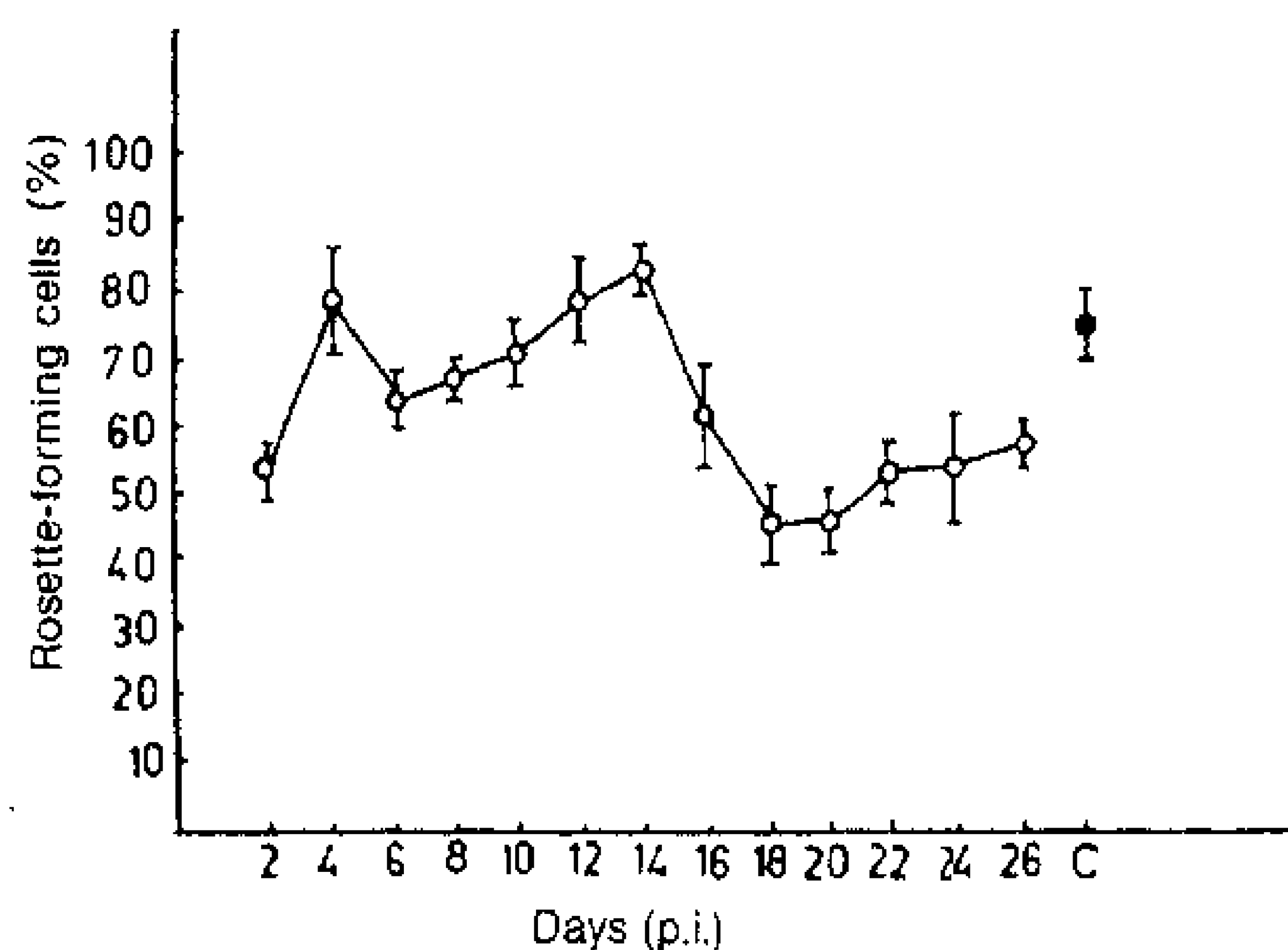

Figure 1. Pattern of attachment of opsonized sheep erythrocytes (EA) by peritoneal macrophages at different time after intraperitoneal inoculation of JEV (O) or in uninoculated control (●). Each value represents the mean±SD from 5 to 7 mice.

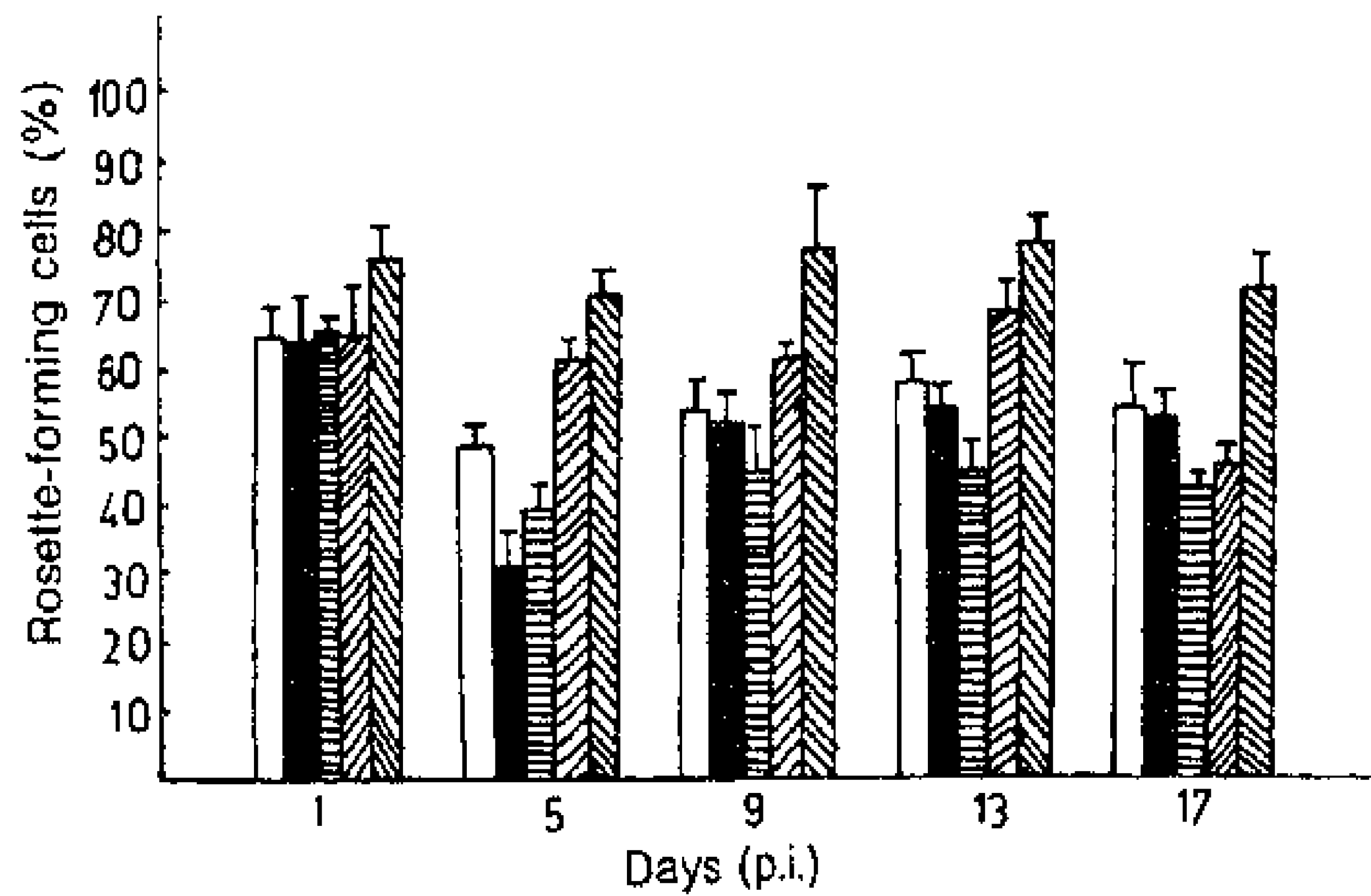

Figure 2. Pattern of attachment of opsonized sheep erythrocytes by peritoneal macrophages on different days after inoculation of TS1 (□); TS2 (■). JEV and TS1 (▤); JEV (▩) or uninoculated control (▨). Mean value ± SD presented.

functions. Figure 2 shows that following Ts_1 cell inoculation 64±6% of macrophages attached EA on day 1 which slightly decreased on day 5 (48±2.6%) and then recovered. A sharp decline in the capacity of macrophages to attach EA (31±7.0%) was observed on day 5 after Ts_2 administration which increased thereafter to 52±1.5% but remained below the control values throughout the study period. In control mice 70–78% of macrophages showed attachment of EA.

In another experiment the combined effect of JEV and Ts_1 on Fc-receptor-mediated attachment of EA by peritoneal macrophages was studied (Figure 2). In mice given JEV and suppressor cells simultaneously, the binding of EA by macrophages was reduced significantly compared to that of mice receiving JEV alone or Ts_1 cells alone on day 5. The values remained low throughout the study period in comparison with controls.

Effect of SF on Fc-receptor-mediated attachment by macrophages

Groups of mice inoculated with SF (1:50) i.p. were sacrificed at different periods and the capacity of macrophage to bind opsonized SRBC through Fc receptor was studied. Figure 3 shows that in the normal control group the percentage of rosette forming Mϕ was 78±6% while that in SF inoculated groups was reduced throughout the study period with the mean value of 49±5%.

The effect on attachment of opsonized SRBC by Mϕ on different days after simultaneous i.p. inoculation of SF (1:50) and JEV was studied. A sharp decline in the proportion of cells binding to opsonized SRBC was seen 11 to 13 days p.i. being 18 to 20%. The Fc-receptor-mediated attachment values recovered gradually (Figure 3).

In vitro effect of SF on Fc-receptor-mediated attachment

In vitro effect of SF on the macrophages to suppress

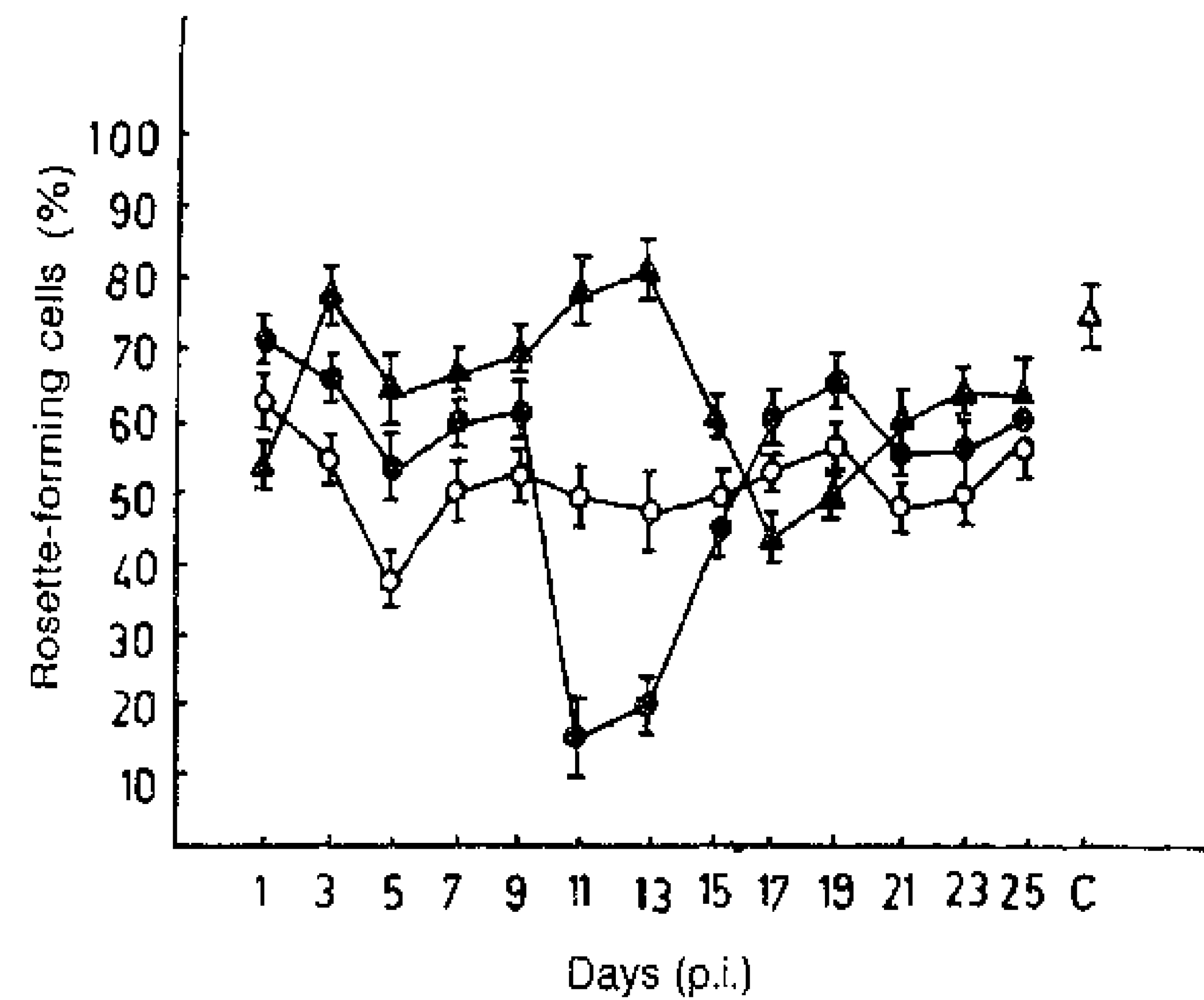

Figure 3. Pattern of attachment of EA on peritoneal macrophages on different days after inoculation of SF and JEV given at the same time (●), or SF (○), or JEV (▲), or uninoculated control (△). Mean value ± SD presented.

their binding capacity for the opsonized SRBC was studied. The Mϕ cell sheet was incubated with different dilutions of SF for 1 h at 37°C and then washed. Table 1 shows that the rosette-forming capacity of Mϕ remained unaltered as there was no difference in the binding capacity of control (84.3±0.98%) and different dilutions of SF-treated macrophages.

Discussion

The results show that during JEV infection, the Fc-receptor-mediated attachment of EA to mouse macrophages was depressed. The macrophages lost their ability to bind EA markedly on day 18 p.i. At later periods this recovered slightly but remained significantly lower than the controls.

The depression of Fc-receptor functions of macrophages can be caused by different mechanisms, viz. immobilization of the receptors[12,13]; selective internalization of macrophage membrane[14]; production of antibody to macrophage surface by host[15]; decreased contents of cyclic AMP-dependent protein kinase[5]; killing or functional damage of macrophages by the

Table 1. *In vitro* effect of SF on Fc-receptor mediated attachment.

Group	Dilution	% positive* ± SD
Normal	—	84.3±0.98
SF	1:20	81.3±3.74
SF	1:50	79.7±0.7
SF	1:100	82.3±1.5

Mϕ monolayers prepared from normal mouse peritoneal fluid were incubated with different dilution of SF for 1 h at 37°C and then assayed for Fc-receptor-mediated binding capacity.
Mean ± SD presented.
*Macrophages with 3 or more than 3 SRBC's attached on it were considered positive.