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Antigen-Specific Helper Activity in Serum of Mice Primed with Sheep Red Cells

I. Definition of the Test System and Comparison with Other Systems

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An adoptive transfer system is described to measure serum helper activity in the primary antibody response to sheep red blood cells (SRBC). Mice injected with a high dose of cyclophosphamide and reconstituted with rabbit anti-thymocyte serum-treated spleen cells were used as recipients. Serum obtained 9 hr after ip injection of normal mice with 2×10^8 SRBC (S(SRBC)) injected i.v. in the recipients caused a significant enhancement of the antibody response to 2×10^7 SRBC. The serum helper activity was not generated in thymectomized animals and could be absorbed from S(SRBC) by normal and formalinized SRBC. The SRBC-specific serum helper activity (SSHA) is heat labile (30 min 56°C) and shows allogeneic restriction. Another test system described in literature for measuring T-cell help *in vivo* was less suited to measure SSHA in the response to 2×10^7 SRBC. A system using normal mice injected with 10^5 SRBC for determining specific immune response-enhancing factor (SIREF), demonstrated SIREF activity in S(SRBC). It did, however, not measure SSHA, as absorption of S(SRBC) with formalinized SRBC did not abolish the activity in that system.

INTRODUCTION

Antibody formation to heterologous red cells in the mouse requires cooperation between T lymphocytes, macrophages, and B lymphocytes both *in vitro* and *in vivo* (1–4). Among the T cells, involved helper, amplifier, and suppressor cells bearing different Lyt-antigens can be distinguished (5, 6). Helper T cells are sensitive to treatment with rabbit anti-thymocyte serum (ATS),[1] whereas amplifier cells are not (6, 7). T–B cell cooperation in the primary antibody response to sheep red blood cells (SRBC) is only possible when the T and B cells are compatible at the H-2 level (8, 9). This phenomenon is called allogeneic restriction. *In vitro* studies of antibody formation to different kinds of antigens (soluble or particulate) suggest that the

[1] Abbreviations used in this paper: ATS, rabbit anti-thymocyte serum; CRBC, chicken red blood cells; CY, cyclophosphamide; i.p., intraperitoneal(ly); i.v., intravenous(ly); *n*, number of animals used; NMS, normal mouse serum; NS, not significant; S( ), serum of mice immunized with or according to; SEM, standard error of the mean; SIREF, specific immune response-enhancing factor; SRBC, sheep red blood cells; SSHA, SRBC-specific serum helper activity.

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activation of B cells involves soluble helper, enhancing (amplifier) and suppressor factors of antigen-specific and nonspecific nature (10, 11). Allogeneic restriction is also reflected in some antigen-specific factors (12). A helper factor, specific for (T, G)-A–L and not genetically restricted (13), was shown to be active *in vivo* in irradiated mice reconstituted with bone marrow cells. Diamantstein and Naher (14) were the first who demonstrated the *in vivo* generation of a soluble product concerned in antibody formation. The factor was present in serum of mice 4 hr after intravenous (i.v.) injection of 10^9 SRBC and enhanced the response to 10^5 SRBC in an antigen-specific way. It acted through the H-2 barrier and was called specific immune response-enhancing factor (SIREF).

Our study was directed to the *in vivo* generation of antigen-specific helper factor(s), which, in analogy to *in vitro* studies, might replace T cells in the primary immune response of mice to SRBC. Serum of mice obtained after intraperitoneal immunization with SRBC was tested for helper activity. As assay system for helper activity we used mice treated with a high dose of cyclophosphamide (15) and reconstituted with spleen cells treated with ATS + C (16) for selective removal of helper T cells (7). A number of characteristics of serum helper activity was investigated. The results obtained with antigen-specific serum helper activity in our test system and in those of others (13, 14) were compared.

MATERIALS AND METHODS

Animals. F_1 (BALB/c × Swiss inbred) mice were bred and maintained in the Laboratory of Microbiology (Utrecht, The Netherlands). In one experiment mice of the parent strains were used. Male mice were used at an age of 12–13 weeks. New Zealand white rabbits were used for the production of ATS.

Cell suspensions. SRBC, stored in Alsever's old solution, were obtained from the National Institute of Public Health (RIV, Bilthoven, The Netherlands). In some experiments formalinized SRBC were used (17).

Chicken red blood cells (CRBC), stored in Alsever's old solution, were kindly provided by Dr. W. Seinen (Institute of Pathology, Veterinary Faculty of the State University of Utrecht, The Netherlands). All red blood cells were washed three times and resuspended in sterile saline before use.

Suspensions of murine spleen cells and IgG_{2B} tumor plasma cells isolated from the BALB/c ascites tumor MOPC 195, were prepared as described earlier (18).

Sera. Mice were i.p. injected with SRBC, CRBC, or saline. At varying hours after the injection blood was withdrawn with capillary tubes from the retro-orbital plexus and allowed to clot for 1 hr at room temperature. The sera obtained: S(SRBC), S(CRBC), and S(saline) were stored at −70°C.

SIREF-Containing serum was prepared by injecting 10^9 SRBC i.v. and collecting the serum 4 hr later (14). This serum was designated S(D-N).

ATS was prepared in rabbits by two i.v. injections of 2×10^8 murine thymocytes with a 2-week interval. Ten days after the last injection the rabbits were bled for serum (19).

Fresh guinea pig serum was used as source of complement (C).

Absorption procedures. As a rule ATS and C were absorbed by incubation of 3 vol of serum with 1 vol of packed IgG_{2B} tumor plasma cells during 20 min at 4°C.

For control experiments mouse sera were absorbed by incubation of 3 vol with 1 vol of packed SRBC, formalinized SRBC, or CRBC.

Assay systems for T-cell help and SIREF activity. Cyclophosphamide treatment of mice was used as a substitute for total body irradiation (15). Cyclophosphamide (CY, Koch Light Laboratories, Colnbrook, Bucks., England) was administered i.p. to animals in a dose of 350 mg/kg body wt (20). Eight hours later they received an i.v. transfer of 2×10^7 syngeneic spleen cells, treated *in vitro* with ATS and C (16). Inguinal lymph node cells of mice primed 6 weeks before with 10^6 SRBC were used as a source of T helper cells (21). Helper activity was tested by simultaneous i.v. injection of ATS-treated spleen cells, 5×10^6 primed lymph node cells, or an indicated volume of one of the sera S(SRBC), S(CRBC), and S(saline) and 2×10^7 SRBC. The numbers of direct anti-SRBC plaque-forming cells (PFC) were determined in the spleens 5 days after transfer and immunization (22, 23).

In the system of Taussig (13) mice received total body irradiation of 850 rads on a teletherapy ^{60}Co unit (Picker, C4M/60). Three days later the animals were reconstituted i.v. with 10^7 normal bone marrow cells and immunized with 2×10^7 SRBC. Serum helper activity was tested by simultaneous injection of 0.2 ml of S(SRBC) or S(saline). The direct splenic PFC response was measured 12 days after immunization (23).

In the system for measuring SIREF activity (14) mice were injected with 0.1 ml of S(D-N) or S(SRBC) and 10^5 SRBC. Controls received 0.1 ml of normal mouse serum (NMS) instead of S(D-N) or S(SRBC), or saline instead of SRBC. The direct splenic PFC response was measured 6 days after immunization.

Thymectomy. Mice, thymectomized under aseptic conditions at an age of 4 weeks, were used 3 months after surgery. Sham-operated animals served as controls.

Statistical analysis. Results have been expressed as the arithmetic mean of n independent observations ± standard error of the mean (SEM). Student's t test

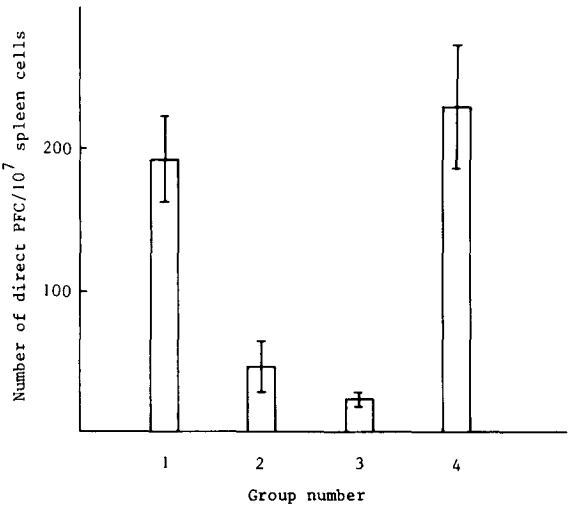

FIG. 1. Operability of the test system for measuring T-cell help. Mice were injected with 350 mg cyclophosphamide/kg body wt. Eight hours later the animals were reconstituted with 2×10^7 syngeneic spleen cells (group 1), ATS-Treated spleen cells (group 2), 5×10^6 peripheral lymph node cells of animals primed i.p. 6 weeks before with 10^6 SRBC (group 3), or a mixture of the latter two (group 4). All animals received i.v. 2×10^7 SRBC simultaneously with the cell transfer. The direct PFC response was determined on Day 5 ($n = 6$ per group, vertical bars indicate the SEM). The differences between columns 1 and 2 and between 2 and 4 are significant ($P < 0.001$).

was performed to analyze the statistical significance of the results. Values of P over 0.05 are considered to be not significant (NS).

RESULTS

Demonstration of T-Cell Help with the Assay System.

Groups of mice were treated with CY (350 mg/kg) and 8 hr later reconstituted i.v. with syngeneic lymphocytes. The following cells or combinations of cells were transferred: 2×10^7 spleen cells as such (group 1) or after treatment with ATS (group 2); 5×10^6 inguinal lymph node cells of primed mice (group 3); or a mixture of 2×10^7 ATS-treated spleen cells and 5×10^6 primed lymph node cells (group 4). The mice received 2×10^7 SRBC simultaneously with the lymphocytes. The numbers of direct PFC in the spleens were determined 5 days after transfer and immunization. Figure 1 shows that ATS treatment of the spleen cells caused a dramatic fall in the numbers of PFC. The response of ATS-treated spleen cells, however, was restored upon addition of primed lymph node cells, while the primed lymph node cells by themselves did not induce a significant response. This demonstrates the operability of the test system to measure T-cell help.

Variations in the Antigen Dose Used for the Induction of S(SRBC)

Mice were i.p. injected with graded numbers of SRBC and blood was collected 9 hr later. The sera were tested in 0.2-ml amounts in the assay system for helper activity. From Fig. 2 it is clear that helper activity is present in serum of i.p.-immunized mice. The optimal dose for its induction was 2×10^8 SRBC.

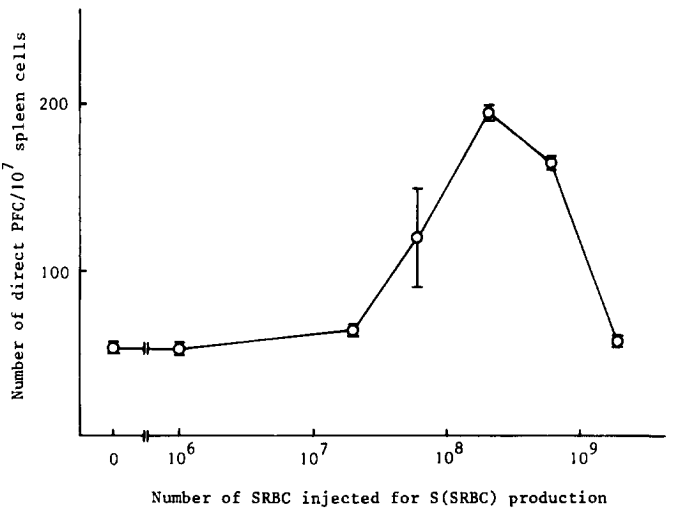

FIG. 2. Antigen dose dependency of serum helper activity 9 hr after i.p. immunization. CY-Injected animals (n = 20 per group) were immunized with 2×10^7 SRBC and transferred with ATS-treated spleen cells and 0.2 ml of serum obtained 9 hr after i.p. immunization of mice with increasing numbers of SRBC. The direct PFC response was measured in the spleen on Day 5 (vertical bars indicate the SEM). The sera obtained after immunization of mice with 2×10^7, 6×10^7, 2×10^8, and 6×10^8 SRBC show significant helper activity ($P < 0.025$, 0.025, 0.001, and 0.001, respectively).

Time Dependence of the Induction of Helper Activity

Mice were i.p. injected with 2×10^8 SRBC and blood was collected at varying hours after the injection. The sera were tested in 0.2-ml amounts in the assay system for helper activity. Although helper activity was already demonstrable 3 hr after immunization, maximal activity was attained 9 hr after immunization (Fig. 3). The S(SRBC), S(CRBC), and S(saline) used in further experiments were prepared from blood collected from mice injected 9 hr before with 2×10^8 SRBC (0.4 ml 2% in saline) or a same amount of CRBC, or saline only.

Dose Dependence of Serum Helper Activity

The dose-dependence of serum helper activity was measured by using graded amounts of S(SRBC) in the assay system for helper activity. S(CRBC) and S(saline) did not show significant activity (Fig. 4). The helper activity of 0.2 and 0.5 ml S(SRBC) did not differ significantly and reached values comparable with those obtained with 5×10^6 lymph node cells of primed mice (compare Fig. 1). In further experiments 0.2 ml of S(SRBC) was used.

Some Characteristics of the Serum Helper Activity

The effects of early thymectomy on the generation of serum helper activity in S(SRBC) and of *in vitro* treatments of S(SRBC) and S(saline) were studied. Early thymectomy prevented the generation of serum helper activity (Fig. 5). Furthermore the helper activity proved to be SRBC-specific, since it was removed from S(SRBC) by absorption with both normal and formalinized SRBC and not by control absorption with CRBC. The SRBC-specific serum helper activity (SSHA) was labile on heating during 30 min at 56°C.

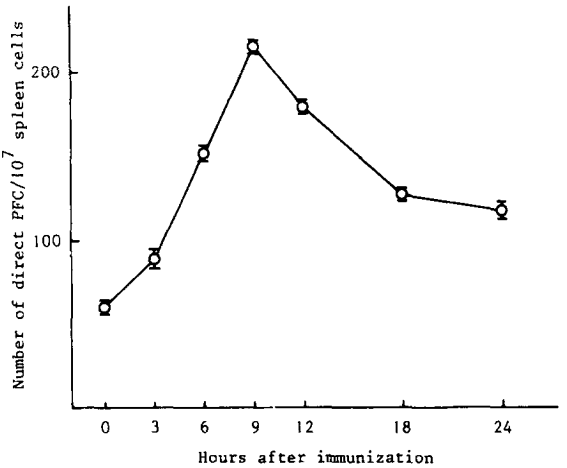

FIG. 3. Time dependence of serum helper activity development after i.p. immunization with 2×10^8 SRBC. CY-Treated mice ($n = 20$ per group) received a transfer of ATS-treated spleen cells and 0.2 ml of serum obtained from mice at different times after i.p. immunization with 2×10^8 SRBC. The cells and serum of the transfer were mixed beforehand with 2×10^7 SRBC. The splenic IgM response was determined on Day 5 (vertical bars indicate the SEM). All values differ significantly from the control value ($P < 0.001$).

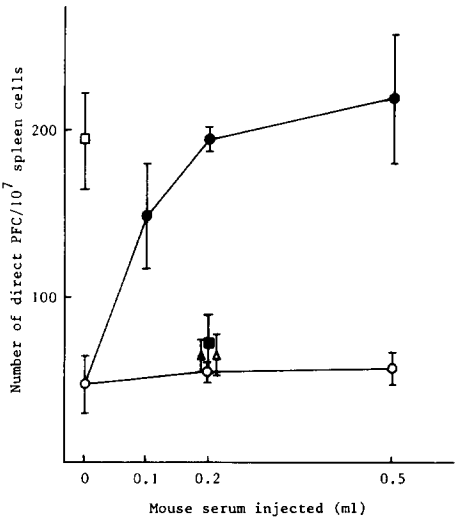

FIG. 4. Dose-dependent helper activity of serum obtained 9 hr after immunization. Mice (n = 6 per group) were injected with CY and reconstituted with ATS-treated spleen cells and increasing doses of S(SRBC) (●) or S(saline (○). A control group received 0.2 ml of S(CRBC) (■). These mice and those reconstituted with untreated spleen cells (□) were immunized with 2×10^7 SRBC. The antibody response on Day 5 was significantly decreased by ATS treatment of spleen cells ($P < 0.001$), but was dose dependently restored by S(SRBC) (P(0.1 ml) < 0.01; P(0.2 ml) < 0.001; P(0.5 ml) < 0.005). No restoration was found upon injection with S(CRBC) and S(saline). The response of nonimmunized mice injected with ATS-treated spleen cells and S(SRBC) or S(saline) is represented by (▲) and (△), respectively.

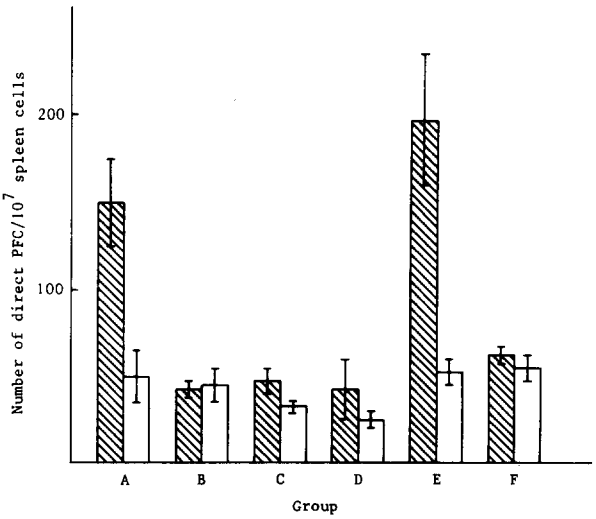

FIG. 5. Characteristics of the serum helper activity. S(SRBC) (▨) and S(saline (□) of normal (A, C–F, n = 10) and of thymectomized mice (B, n = 5) were tested in 0.2-ml amount for helper activity in CY-treated mice reconstituted with ATS-treated spleen cells, and immunized with 2×10^7 SRBC. The sera of the groups C–F were pretreated in different ways: (C) absorbed with normal SRBC; (D) absorbed with formalinized SRBC; (E) absorbed with CRBC; (F) heated for 30 min at 56°C. The decreases in antibody responses on Day 5 (vertical bars indicate the SEM) for S(SRBC) in the groups B–D and F are statistically significant ($P < 0.001$).

TABLE 1

Allogeneic Restriction of SRBC-Specific Serum Helper Activity[a]

Serum donor	Serum	Recipient mice	
		BALB/c	Swiss inbred
BALB/c	S(SRBC)	94.2 ± 13.4[b,**]	37.6 ± 9.9
BALB/c	S(saline)	46.4 ± 5.9	36.5 ± 12.1
Swiss inbred	S(SRBC)	64.4 ± 8.3	56.7 ± 18.8*
Swiss inbred	S(saline)	56.0 ± 9.4	25.2 ± 6.8

[a] Donor mice (n = 7–21) were injected i.p. with 2×10^8 SRBC or saline. Nine hours later the animals were bled for serum. The pooled sera were tested for SSHA in groups of 5–15 recipient mice.

[b] No of direct PFC/10^7 spleen cells Day 5 after transfer and immunization.

* $P < 0.05$.

** $P < 0.005$.

Allogeneic Restriction

For further discrimination the possible genetically restrictedness of SSHA was investigated. S(SRBC) and S(saline) were prepared in the parent BALB/c (H-2^d) and Swiss inbred (H-2^s) strains. The sera were tested for SSHA in test systems using syngeneic mice and mice of the other strain. Table 1 indicates that S(SRBC) originating from BALB/c mice only displayed SSHA in the BALB/c reconstitution system and the same was observed for the Swiss inbred strain. The response, however, within both parent strains was considerably lower than in the F_1 generation (compare Table 1 and Figs. 4 and 5).

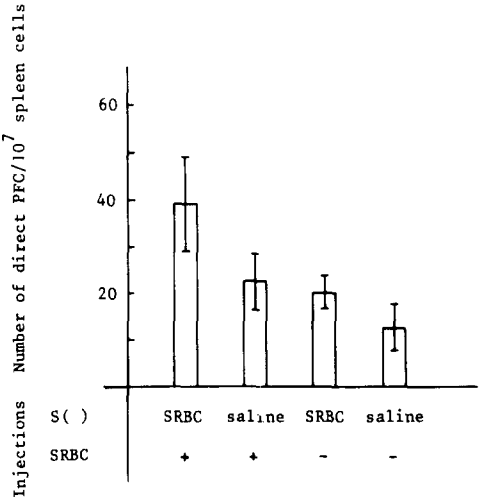

FIG. 6. Serum helper activity in irradiated, bone marrow-reconstituted animals. Mice (n = 5 per group) were irradiated and received 10^7 syngeneic bone marrow cells 3 days later. The animals were injected with 2×10^7 SRBC and 0.2 ml of S(SRBC) or S(saline). Control animals were not immunized. The enhancement of the antibody response on Day 12 (vertical bars indicate the SEM) by S(SRBC) vs S(saline) is NS.

Other Test Systems to Measure T-Cell Help or Amplification

To compare SSHA with other factors displaying helper or enhancing activity *in vivo*, S(SRBC) and S(saline) were introduced in the systems described by Taussig (13) and Diamantstein and Naher (14).

Figure 6 shows that injection of S(SRBC) into irradiated and bone marrow-reconstituted mice together with 2×10^7 SRBC resulted in a slightly, but not significantly, enhanced antibody response on Day 12.

Injection of S(SRBC) or S(D-N) in normal mice greatly enhanced the antibody response to 10^5 SRBC on Day 6 (Fig. 7). The enhancing activity was only partially removed by absorption of S(SRBC) with formalinized SRBC (Fig. 8).

DISCUSSION

This study deals with the possible *in vivo* generation of T-cell-dependent, antigen-specific serum helper activity in the primary antibody response of mice to SRBC. The system generally in use to study T–B cell cooperation employs lethally irradiated mice reconstituted with bone marrow (1, 3). This system was also used by Taussig (14) in studying the *in vivo* activity of an antigen-specific T-cell factor, raised *in vitro*, in the response to (T, G)-A–L. We applied CY treatment for immunological depletion of animals. The use of CY instead of irradiation as a tool in transfer studies has been introduced by Santos and Owens (15) and the suitability of this system for the study of activities of normal cells (24, 25) and of cells from

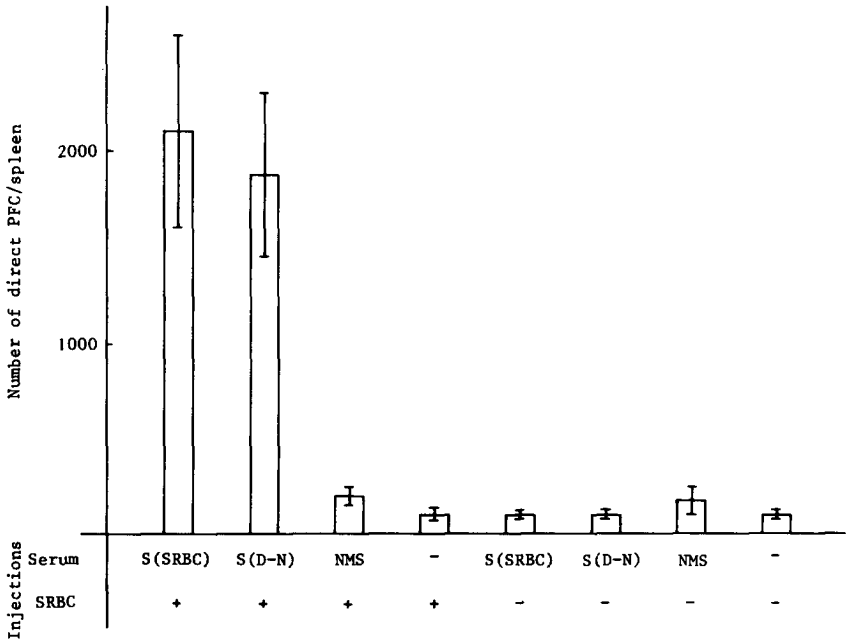

FIG. 7. Serum-enhancing activity in the response to 10^5 SRBC. Mice (n = 5–18) were i.v. injected with 0.1 ml of S(SRBC), S(D-N), NMS, or saline and immunized with 10^5 SRBC. Controls received saline instead of SRBC. The antibody response was measured on Day 6. Vertical bars indicate the SEM. The enhancement of the response to 10^5 SRBC by S(SRBC) and S(D-N) is statistically significant ($P < 0.01$ and $P < 0.005$, respectively).

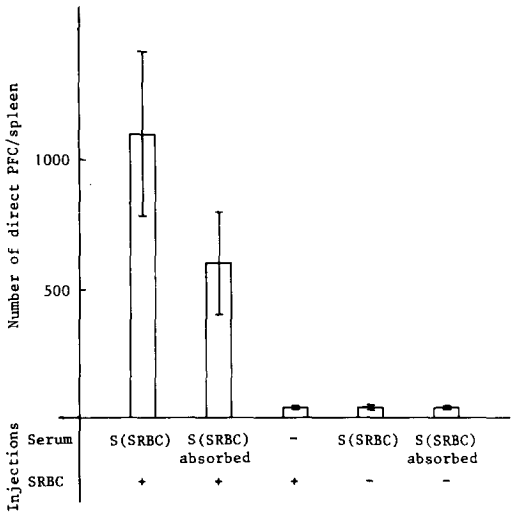

FIG. 8. Effect of absorption of helper positive serum on its enhancing activity in the response to 10^5 SRBC. S(SRBC) as such or after absorption with formalinized SRBC was tested for SIREF activity (n = 5 per group). Control animals received saline instead of serum or SRBC. The decrease in enhancing activity of absorbed S(SRBC) vs untreated S(SRBC) is NS (vertical bars indicate 1 SEM).

busulphan-(26) and CY-(20, 27) treated donors has been confirmed. After CY had been eliminated from the circulation (28), the mice were reconstituted with syngeneic lymphocytes. As we studied the splenic antibody response, spleen cells were used as a source of B cells. The spleen cells were incubated beforehand with rabbit ATS + C, which was reported to be suitable for the elimination of helper (and suppressor), but not of amplifier T cells (6, 7, 29). The immunizing dose of 2×10^7 SRBC causes a suboptimal response, but at this dose the antibody response to SRBC is more thymus dependent than at higher doses (30). The workability of the CY reconstitution system to measure T-cell help has been demonstrated (16) and was confirmed by our experiments (Fig. 1). The system seemed moreover suited to estimate serum helper activity (Fig. 2). Helper activity was optimal in serum as early as 9 hr after i.p. immunization of mice with 2×10^8 SRBC (Figs. 2 and 3). Similar early events in the *in vivo* response to SRBC at the cellular level were described by others (31, 32), while a soluble enhancing factor (SIREF) could be detected as early as 4 hr after i.v. immunization of mice with 10^9 SRBC (14). The failure to produce SSHA in thymectomized mice suggests that T cells are at least required for its generation. Also the SIREF production was found to be T-cell dependent (14). Furthermore both factors are antigen-specific. The soluble help was hence called SRBC-specific serum helper activity (SSHA). In our test system 0.2 ml of S(SRBC) increased the response of splenic B cells to 2×10^7 SRBC 3 to 5-fold (extreme values of 2- and 28-fold were found), but displayed no reactivity in the absence of antigen (Fig. 4). In the parent strains, however, SSHA raised the response of syngeneic B cells only by a factor 2, whereas the allogeneic combinations of SSHA and B cells did not cooperate at all (Table 1). This suggests, first, that the F_1 generation is more apted to study SSHA and, second, that SSHA is genetically restricted. The latter means that SSHA differs from the factor described by Taussig (13) and from SIREF (14). The primary immune response to SRBC and

other particulate antigens is genetically restricted at the level of interaction between T and B cells (8, 9) whereas the secondary response to soluble antigens is restricted at the macrophage–T-cell level (33–37). Therefore, the allogeneic restriction of SSHA, which was raised and tested in a primary system, suggests a direct action on B cells. SIREF is more likely to act on T cells (14).

The other system to study cellular or soluble T-cell help was less suited, at least in unmodified form, to measure SSHA. In the system described by Taussig (13) using irradiation and bone marrow reconstitution, S(SRBC) caused a nonsignificant enlargement of the response by only a factor 1.7 (Fig. 6). The response, however, was measured 12 days after immunization, which may be the optimal time to study specific T-cell help to (T, G)-A–L, but not for measuring SSHA in the primary response to SRBC. In the system of Diamantstein and Naher (14), which measures serum enhancing activity in the IgM response to 10^5 SRBC, S(SRBC) was very active. It enhanced the response even slightly more than S(D-N). The enhancement of the IgM response could, however, not be ascribed to SSHA, as absorption of S(SRBC) with formalinized SRBC resulted in complete loss of SSHA (Fig. 5), but not of the enhancing activity measured in Diamantstein's system (Fig. 8). SSHA differed further from SIREF in allogeneic restriction. These two observations suggest that there are two factors generated early in the response to 2×10^8 SRBC: SSHA and SIREF, which might present the humoral principles of helper and amplifier cells involved in the primary antibody response to SRBC. In that construction SSHA would be a product of the helper T cell which was reported to be antibody-independent (38, 40), whereas SIREF would be formed by the immunoglobulin-dependent amplifier cell (38–42).

In conclusion it can be said that the test system we describe is useful to study this particular type of soluble T-cell help. The active principle, SSHA, is distinguishable from other helper or enhancing factors with demonstrated *in vivo* activities. Our results suggest that SSHA is for its generation dependent on the ATS-sensitive helper T cell. It acts most probably on B cells and might be the *in vivo* correlate of antigen-specific, T-cell factors generated and tested in *in vitro* systems (43, 44). Further study will be directed to the preparation of an antiserum against SSHA and a more extensive characterization of the factor(s) involved.

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