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Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factor(s)

(lymphocyte activation/mixed lymphocyte culture/ultraviolet radiation)

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ABSTRACT **The ability of three cultured mouse tumor lines to stimulate a cytotoxic response in 5-day cultures of allogeneic lymph node cells was studied with a ^{51}Cr release assay. Two lines of mesenchymal origin, P815 and EL-4, were found to be highly stimulatory, whereas the third cell line, CaD2, a mammary gland epithelial tumor, did not stimulate over a wide range of cell concentration. CaD2 cells were shown to contain major antigens similar to those of P815 cells by the specific lysis of both cells by lymphocytes activated to H-2^d-bearing peritoneal cells. UV-irradiated P815-cells, like γ-irradiated CaD2 cells, did not stimulate a cytotoxic response, but both cell lines were found to stimulate a full and specific response to allogeneic lymph node cells if these mixed cultures were supplemented with a supernatant harvested from concanavalin A-stimulated spleen cells.**

We have postulated that a specialized stimulator cell designated as S^+ is required for T cell activation (1). Antigen presented on the surface of the S^+ cell should facilitate the interaction of the stimulator cell with the potentially responsive T cell clone. Thus, T cell activation was thought to require antigen recognition in conjunction with the delivery of an inductive stimulus from the S^+ cell. The production of the inductive agent by the stimulator cell or its delivery to the responsive T cell must usually require metabolic activity of the S^+ cell (refs. 2 and 3; for exception, see ref. 4).

Allogeneic T cell activation occurs when S^+ cells interact with allogeneic T cells. In this case the antigen is built into the surface of the S^+ cell and the intervention of exogenous antigen is not required for T cell activation. Thus, by using different cell types as stimulators for allogeneic T cells, it is possible to determine the S phenotype (S^+ or S^-) of the cell under investigation. The available evidence indicates that cells of the lymphocyte–macrophage class may express the S^+ phenotype (3, 5, 6), while tissue parenchymal cells appear to lack stimulator activity (5, 7), and it is for this reason that passenger leukocytes play a role of over-riding importance in the activation of a specific immune response to tissue allografts.

In this paper we examined the stimulator characteristics of three tumor cell lines. Two of these were of mesenchymal origin and could be shown to be S^+. The other, an epithelial cell, was nonstimulatory and thus S^-. The presence of antigen on the S^- cells could be demonstrated by lysing them with cytotoxic cells activated with S^+ cells of the same genotype and by producing specific stimulation with a combination of S^- cells and a supernatant of spleen cells incubated with concanavalin A (Con A).

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MATERIALS AND METHODS

Mice. DBA/2 or BALB/c (H-2^d), C57Bl/6 (H-2^b), and C3H and CBA (H-2^k) strains were used at 8–12 weeks of age.

Cell Lines. The P815 mast cell tumor of the DBA/2 mouse was maintained in tissue culture in Dulbecco's modified Eagle's medium (Grand Island Biological Co. H-16), supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin, and neomycin, each 100 μg/ml).

The EL-4 lymphoma used as a stimulator cell was a cultured cell line obtained from a C57Bl/6 mouse ascites tumor. This EL-4 was maintained in H-16 supplemented with 10% FCS and antibiotics. EL-4 cells used for ^{51}Cr release assays were ascites tumor cells obtained from the peritoneal cavity of C57Bl/6 mice.

Carcinoma D_2 (CaD2), a mammary carcinoma of DBA/2 mice, was obtained from Jackson Laboratories and maintained in DBA/2 mice as a solid tumor requiring passage by subcutaneous inocultation of 1-mm fragments every 2 weeks. An *in vitro* cell line growing as a monolayer with a doubling time of approximately 12 hr was obtained by incubating small fragments of the tumor at 37° with 0.1% trypsin for 30 min in Puck's saline A (Grand Island) and then washing and culturing the cells in Eagle's medium (Grand Island F-15), supplemented with 1 mM pyruvate, antibiotics, and 10% FCS. A single cell suspension was obtained for passage, for stimulating lymphocytes in culture, and for labeling with ^{51}Cr by incubating the monolayers with 0.1% trypsin for 20 min. The cells were then washed twice with F-15 and counted. The CaD2 cells used in these experiments had been grown *in vitro* by weekly transfer for more than 3 months.

Mouse Peritoneal Cells were obtained by washing the peritoneal cavity of normal mice with F-15 containing heparin at 20 units/ml.

Mouse Spleen Cell Suspensions were obtained by gently teasing apart the spleen in F-15. Larger particles were allowed to settle out of the cell suspension and the latter was then washed and reconstituted in the tissue culture medium used in *in vitro* assays.

Lymph Node Cell Suspensions were made in a similar manner from mouse mesenteric, axillary, and inguinal lymph nodes.

Leukocyte Suspensions from Guinea Pig and Bovine Spleens and from Human Peripheral Blood Buffy Coat were washed with Hanks' solution, centrifuged, and resuspended in

Abbreviations: T cell, thymus-derived cell; S^+, phenotype of cell with capacity to stimulate allogeneic lymphocytes; F-15, Eagle's medium; H-16, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; C.U., cytotoxic units; Con A, conanavalin A; CSCS, Con A-induced spleen cell supernatant.

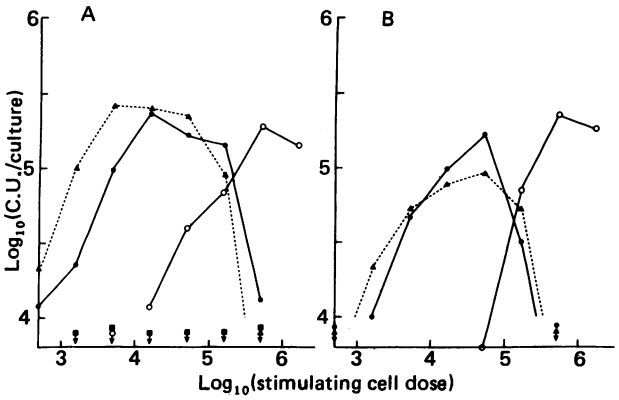

FIG. 1. (*A*) Cytotoxic cell responses of C57Bl/6 (H-2^b) lymph node cells cultured with the following H-2^d cells: ○, γ-irradiated (750 R) BALB/c spleen cells; ●, γ-irradiated (750 R) BALB/c peritoneal cells; ▲, γ-irradiated (5000 R) P815 mast tumor cells; ■, γ-irradiated (5000 R) CaD2 mammary tumor cells. (*B*) Cytotoxic cell response of BALB/c (H-2^d) lymph node cells cultured with the following H-2^b cells: ○, γ-irradiated (750 R) C57Bl/6 spleen cells; ●, γ-irradiated (750 R) C57Bl/6 peritoneal cells; ▲, γ-irradiated (5000 R) EL-4 lymphoma cells.

30 volumes of 0.85% NH_4Cl. The suspensions were incubated 15 min at 37° to lyse erythrocytes and then washed twice with Hanks' solution.

Irradiation of Stimulator Cells. In some experiments stimulator cells were given γ-irradiation from a ^{60}Co source before *in vitro* culture. In some cases the tumor cells were irradiated for 4 min with a 30-W General Electric germicidal G30T8 lamp (ultraviolet) at an intensity of 960 μW/cm^2 in the 230- to 270-nm range. For irradiation 1.5 × 10^6 cells were placed in a coverless 35-mm petri dish in 2 ml of F-15 + 10% FCS.

***In Vitro* Generation of Cytotoxic Cells.** Cytotoxic cells were generated by culturing irradiated stimulator cells with responding lymph node cells in multiwell tissue culture plates (Falcon no. 3008). Various doses of stimulating cells were mixed with 10^6 responding cells in triplicate 1-ml cultures containing F-15 supplemented with 0.1 mM 2-mercaptoethanol, 10% FCS, and antibiotics. Cell cultures were maintained in an atmosphere of 8.5% O_2/79.5% N_2/12% CO_2. This is a high-altitude modification of the 7% O_2/83% N_2/10% CO_2 gas mixture used to maintain such cultures at around sea level. Cell cultures were rocked in a Bellco rocking platform throughout the assay. Rocking was found to increase the efficiency of stimulation at low stimulator cell densities. At the completion of the culture period, which was routinely 5 days, cells were harvested for the cytotoxicity assay.

^{51}Cr Labeling of Target Cells was carried out by incubating 1 ml of the P815 target cells, at a density of 5 × 10^6 cells per ml in F-15 supplemented with antibiotics but without FCS, with 100 μCi of sodium [^{51}Cr]chromate. After an incubation period of 1 hr the cells were washed with three changes of F-15 and finally resuspended at a cell density of 1 × 10^6/ml; 0.1 ml volumes of this cell suspension were used in the cytotoxicity assay.

Cell-Mediated Cytotoxicity. At the completion of the *in vitro* activation, cells from three replicate wells were mixed and resuspended in 1 ml of fresh F-15 supplemented with 10% FCS and antibiotics. Serial 2-fold dilutions of these cell preparations were then prepared in the same medium. From these dilutions, 0.1-ml volumes were then mixed with 0.1 ml of the ^{51}Cr-labeled target cell suspension (10^5 cells) in Linbro multidish plates. Each cell dilution was assayed in duplicate cultures. Details of the 4-hr assay and estimation of the number of cytotoxic units (C.U.) have been previously described (7). Cytotoxic activity is usually expressed as log_{10} (C.U./culture) and replicate assays have a standard deviation of 0.06 log_{10} units. One C.U. is defined as the amount of cytotoxic activity required to lyse one target cell in 4 hr under standard conditions.

Table 1. Cytotoxic activity of H-2^d-reactive cells on P815, CaD2, and EL-4 target cells*

Target cells	Lysis, cpm		Cytotoxic/ target cells	% specific ^{51}Cr release
	Water	Medium		
10^5 P815	8,460	708	4:1	81
			2:1	44
			1:1	23
2 × 10^4 CaD2	6,270	1,130	20:1	50
			10:1	32
			5:1	15
10^5 EL-4	4,310	1,526	20:1	<5

* Prepared in 5-day cultures of 10^6 C57Bl/6 lymph node cells and 10^6 γ-irradiated DBA/2 spleen cells.

Preparation of Con A-Induced Spleen Cell Supernatant (CSCS). Spleen cells or human buffy coat leukocytes were suspended at 10^7 per ml of F-15 with 0.1 mM 2-mercaptoethanol but no serum. A solution of Con A at a concentration of 10 μg/ml in F-15 + 2-mercaptoethanol was sterilized by filtration. Eight milliliters of spleen cell suspension and 8 ml of Con A solution were incubated in each 250-ml Falcon plastic bottle (lying on its side) for 2 hr at 37°. The supernatant and a few nonadherent cells were removed and the adherent cells were washed carefully with three changes of 10 ml of Hanks' balanced salt solution. Sixteen milliliters of F-15 with 2-mercaptoethanol was added to the cell monolayer and the mixtures were incubated at 37° for 16–18 hr. The supernatant was mixed with FCS, 1 ml for each 100 ml, and concentrated 10-fold over a PM-10 Diaflo membrane. The concentrate was sterilized by filtration and stored frozen. This method is only slightly modified from that of Pick and Kotkes for producing migration inhibitory factor (8).

RESULTS

Stimulation Characteristics of Normal and Oncogenic Cells. Preliminary studies showed that cytotoxic cell development in lymph node cultures stimulated by normal and oncogenic S^+ cells displayed similar kinetics. Cytotoxic activity reached a detectable level on the fourth day of culture, rose to a maximum by day 5, maintained this level through the sixth day of culture, and then declined. Unless otherwise stated all cytotoxic activities in the following studies were assayed on the fifth day of culture.

Fig. 1 shows the stimulation characteristics of three tumor lines and two normal cell populations for allogeneic lymph node cells. The tumor cells of mesenchymal origin (P815 and EL-4) showed a stimulation profile similar to that of normal peritoneal cells. Thus, these tumors express the S^+ phenotype of normal leukocytes. On the other hand, the epithelial tumor CaD2 showed no stimulator activity over the wide dose range tested and is therefore classified as an S^- cell line. This failure of CaD2 to stimulate allogeneic lymphocytes was not due to a lack of recognizable H-2 antigen on the surface of this tumor line; C57Bl/6 lymphocytes activated against the H-2^d antigens on

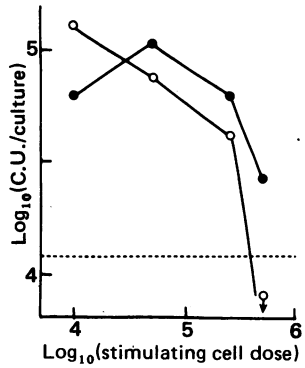

FIG. 2. Cytotoxic cell responses of C57Bl/6 lymph node cells cultured with various numbers of DBA/2 peritoneal cells. O, Incubated 20 min with 0.1% trypsin and washed; ●, untreated. The broken line shows the response of lymph node cells alone cultured in the presence of CSCS.

irradiated DBA/2 spleen cells were cytotoxic for both ^{51}Cr-labeled CaD2 and P815 tumor cells (Table 1). At the highest cell density tested the activated C57Bl/6 cells showed no significant cytotoxicity (<5% lysis) against ^{51}Cr-labeled EL-4 target cells. Clearly, the S^- phenotype of the CaD2 tumor is not due to the lack of recognizable antigen on the surface of this cell line. The failure of CaD2 cells to stimulate is also not due to their treatment with trypsin, because trypsin treatment did not adversely effect the stimulatory capacity of macrophages (Fig. 2).

Con A-Induced Spleen Cell Supernatant (CSCS) Facilitates T Cell Activation by S^- Tumor Cells. Fig. 3 shows that UV irradiation of P815 inactivated these cells' capacity to stimulate allogeneic lymph node cells. The addition of CSCS to these cultures resulted in the generation of high levels of cytotoxic activity. Lymph node cells alone, cultured for 5 days in the presence of CSCS, showed a low but significant degree of lysis (4.5%) when assayed against P815 target cells. The level of cytotoxic activity generated by UV-irradiated P815 cells in the presence of CSCS was significantly higher than that generated

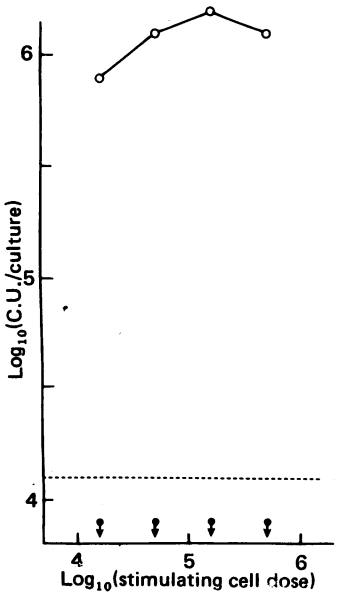

FIG. 3. Cytotoxic cell response of C57Bl/6 lymph node cells, cultured with UV-irradiated (960 $\mu W/cm^2$ in the 230- to 270-nm range) P815 mast cell tumor cells: ●, response in the absence of CSCS; O, response in the presence of CSCS. The broken line shows the response of lymph node cells alone cultured in the presence of CSCS.

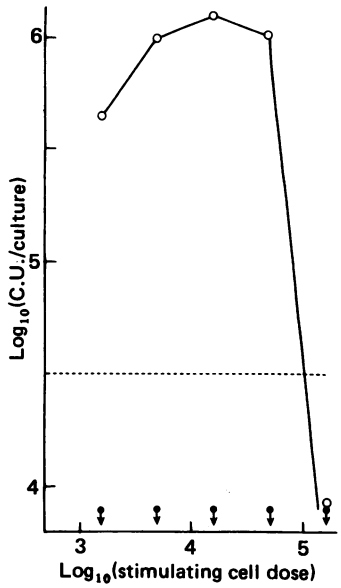

FIG. 4. Cytotoxic cell response of C57Bl/6 lymph node cells cultured with γ-irradiated (5000 R) CaD2 mammary tumor cells: ●, response in the absence of CSCS; O, response in the presence of CSCS. The broken line shows the response of lymph node cells alone cultured in the presence of CSCS.

by γ-irradiated P815 cells in the absence of CSCS (Fig. 1) and the cytotoxic activity generated was specific for the H-2^d antigen on the UV-irradiated cell; no significant lysis above the CSCS background was observed when cells activated in this way were assayed against ^{51}Cr-labeled EL-4 target cells.

CaD2 is a cell line that is constitutively S^-, and γ-irradiated CaD2 cells were unable to stimulate allogeneic T cells (Fig. 1). Fig. 4 shows the cytotoxic cell response obtained when γ-irradiated CaD2 cells were cultured with C57Bl/6 lymph node cells in the presence of CSCS. The addition of CSCS activated a strong cytotoxic cell response to the H-2^d antigens carried on the CaD2 tumor; cytotoxic cells generated in these cultures were assayed against P815 target cells.

At the high stimulator dose (above 10^5 cells per culture) ad-

Table 2. Cytotoxic response of C57Bl/6 lymph node cells (LNC) to UV-treated CaD2 and P815 cells following addition of CSCS at various times

Cells cultured × 10^{-5}			Log_{10}(C.U.*/culture) on day 5 with CSCS added on day					
C57Bl/6 LNC	UV P815	UV CaD2	0	1	2	3	4	Not added
			Experiment 1					
10	1.6	—	5.9	5.9	5.8	5.8	5.0	4.8
10	—	1.6	5.7	5.0	4.3	<3.9	NT	<3.9
10	—	—	4.3	NT	NT	NT	NT	<3.9
			Experiment 2					
10	1.6	—	5.7†	5.6	<3.7	<3.7	NT	<3.7
10	—	1.6	6.2	5.9	4.4	<3.9	<3.9	<3.9
10	—	—	4.6	NT	<3.9	NT	NT	<3.9

NT = not tested.

* When 3/10 of a culture is assayed, 6 log_{10}C.U. is equivalent to 95% specific lysis; 5 log_{10}C.U., to 26% lysis; and 4 log_{10}C.U., to 3% lysis.

† Cytotoxic activity in similar cultures was destroyed by anti-θ serum plus complement. Other cultures were not so tested.

Table 3. Strain specificity of CSCS

Source of CSCS	Dilution of CSCS in culture	Log_{10}(C.U./culture) with various stimulating cells* 10^5 UV-P815	10^5 UV-CaD2	None
C57Bl/6 spleen	1:10	6.1	6.3	4.4
	1:30	5.5	5.3	<3.9
	1:100	5.1	4.9	<3.9
CBA spleen†	1:10	6.1	5.9	4.9
	1:30	5.4	5.0	4.1
	1:100	4.6	4.5	<3.9
C3H	1:10	6.3	6.2	4.7
	1:30	NT	5.4	4.2
	1:100	NT	4.7	<3.9
None added	—	<3.9	<3.9	<3.9

NT = not tested.

* Responding cells were 10^6 C57Bl/6 lymph node cells, the target cells were ^{51}Cr-labeled P815 cells.

† Specific lysis of labeled EL-4 cells was less than 3% (3.9 log_{10}C.U.) in all tests with CBA CSCS.

dition of CSCS did not result in the generation of a cytotoxic response. This phenomenon is probably due to a suppressive effect of the metabolically active γ-irradiated CaD2 cells at high cell densities. With both UV-irradiated CaD2 and P815 the high dose suppression seen with γ-irradiated cells is eliminated (compare Figs. 1 and 3).

CSCS Acts Early and Late in Lymphocyte Stimulation. If CSCS is required for the first step of T cell activation, it would be essential for this material to be present in the culture medium at the time antigen is presented to the responsive cells. If, on the other hand, this factor is merely an amplifier that expands ongoing proliferative responses initiated by the stimulator cell, then its addition after 1–2 days of culture might be expected to have the same final effect.

Table 2 shows the effect of adding CSCS at various times to cultures of C57Bl/6 lymph node cells and either UV-treated P815 or UV-treated CaD2. With UV-treated P815 an enhancing effect could be obtained by adding CSCS as late as day 3 if the P815 were incompleteley inactivated and still produced detectable cytotoxicity (experiment 1). Otherwise (experiment 2) CSCS had to be added on day 0 or 1. The maximal response with CaD2 was obtained when the CSCS was added on day 0. When the CSCS was added on day 1 the response was ½ to ⅕, and a barely significant response above background levels was obtained when the CSCS was added on day 2.

CSCS Is Not Strain Specific. To determine the strain specificity of the CSCS, preparations generated from CBA, C3H, or C57Bl/6 spleen cell cultures were used to activate the cytotoxic cell response in cultures containing UV-irradiated P815 or CaD2 (10^5 cells/culture) and C57Bl/6 lymph node cells (10^6 cells/culture). Each preparation of CSCS was treated at three dilutions. The results of this experiment, shown in Table 3, indicate that there is no strain specificity associated with this factor.

CSCS Is Species Specific. To determine species specificity of CSCS, preparations of UV-treated P815 cells were incubated with mouse lymph node cells alone or in the presence of CSCS from four species (Table 4). Only mouse CSCS provided the necessary stimulation. To complement this finding guinea pig, bovine, and human lymphocytes were incubated with γ-irradiated P815 cells alone or with mouse CSCS or homologous

Table 4. Species specificity of CSCS

Responding cell	Treatment of P815	Log_{10}C.U. with various CSCSs None	Mouse	Guinea pig	Bovine	Human
Mouse L.N.	UV	<3.9	5.8*	<3.9	<3.9	<3.9
Guinea pig L.N.	γ-ray	<3.9	4.9	5.3	NT	NT
Bovine L.N.	γ-ray	<3.9	<3.9	NT	4.7*	NT
Human P.B.	γ-ray	<3.9	<3.9	NT	NT	4.7
Mouse L.N.	γ-ray	5.5	NT	NT	NT	NT

L.N. is lymph node, P.B. is peripheral blood, NT is not tested. For comparison of C.U. with percent lysis see Table 2 footnote.

* Mouse and bovine responding cells incubated with homologous factor without added P815 cells showed a low level of cytotoxicity for this target (5–10% lysis). Guinea pig and human cells showed no detectable lysis in these antigen controls.

CSCS. None of the cells responded to P815 cells alone and all responded in the presence of homologous CSCS better than with mouse CSCS. Human and bovine lymphocytes showed no detectable response in the presence of mouse CSCS.

DISCUSSION

The capacity of tumor cells to activate allogeneic cytotoxic cells *in vitro* is dependent on both the nature of the tumor cell and its metabolic activity. In this study, tumor lines of mesenchymal origin (the EL-4 lymphoma and the P815 mastocytoma) showed stimulating activity (Fig. 1). These tumors, following γ-irradiation to prevent cell proliferation, had stimulation characteristics that were similar to those of γ-irradiated peritoneal cells obtained from mice of the same H-2 type. The stimulation profile of both normal and oncogenic stimulator cells passed through a maximum, and a marked suppression of the cytotoxic cell response was seen at high stimulator cell densities. In the case of peritoneal cells, this suppression has been shown to result from the production of arginase by the peritoneal cells, which depletes the medium of an essential amino acid required for lymphocyte differentiation (9). The epithelial tumor, CaD2, showed no stimulating activity when tested over a wide dose range. This tumor was specifically lysed by cytotoxic cells activated to the $H\text{-}2^d$ antigens of the DBA/2 mouse strain (Table 1), indicating that the failure of this cell line to stimulate was not due to a lack of recognizable antigen on the surface of this tumor. Tumor lines can therefore be classified as S^+ or S^- on the basis of their capacity to activate allogeneic cytotoxic cells *in vitro*. The S^+ phenotype of the P815 tumor was inactivated by UV irradiation, and, in this respect, these cells behaved similarly to normal lymphoid stimulating cells (2).

Neither P815 cells nor EL-4 cells express detectable levels of Ia antigens on their surfaces (ref. 10; I. F. C. McKenzie, personal communication). Thus, Ia antigen recognition is not a requirement for the activation of allogeneic cytotoxic cells and is therefore not a necessary characteristic of the S^+ phenotype. This finding is consistent with other reports that I-region incompatibility is not a requirement for cytotoxic cell activation (11). However, it is true that Ly 1^+ helper cell activation by I-region-incompatible cells can augment the cytotoxic cell response to H-2 K or D antigens (12).

The addition of CSCS to cultures of S^- cells (either γ-irradiated CaD2 or UV-inactivated P815) and allogeneic (C57Bl/6) lymph node cells resulted in the generation of specific cytotoxic cell response to H-2 antigens on the surface of the S^- cells (Figs. 3 and 4). These S^- cells therefore possess the antigen required for recognition by allogeneic lymphocytes but

lack a further requirement for T cell activation that is provided by the CSCS. C57Bl/6 lymphocytes cultured alone in the presence of CSCS showed a low but significant level of cytotoxic activity against P815 target cells. The P815 target cells for these assays were maintained *in vitro* in medium supplemented with 10% FCS, and it is possible that this low level of cytotoxicity may represent a response to calf serum proteins present in the lymphocyte culture medium.

One interpretation of these experimental findings is that T cell activation has a two-signal triggering mechanism and that S^+ cells provide both antigen (signal 1) and a source of the second nonspecific signal required for T cell activation; either signal alone is insufficient for T cell activation (3, 13). According to this model, UV irradiation of S^+ cells would convert them to the S^- phenotype by inactivating their metabolic activity and so inhibiting the generation of the second signal by these cells. CSCS would facilitate the response to UV-irradiated cells by providing an exogenous source of the second signal. Another possible interpretation is that the role of antigen is to focus or concentrate the single nonspecific signal on the responding cell. This would explain why antigen alone produced no activation but the nonspecific factor alone produced a low but definite level of activation.

It is possible that the nonspecific signal acts indirectly through Ly 1^+ helper cells present in either the responding lymphocyte population or in the Con A-stimulated spleen cells (14). According to this model, CSCS either causes nonspecific activation of helper cells or itself contains the nonspecific helper factor. In any case, it would seem likely, in the light of these experiments, that metabolic activity is required for the generation of the nonspecific signal and that this process may be activated by mitogens, or in the case of resting lymphocytes may be activated during the early phase of mixed lymphocyte culture, as suggested by Davidson (15).

Paetkau *et al.* (16) have postulated that CSCS provides a costimulator for T cell activation. The finding that this factor has an early effect, i.e., must be present in cultures during the early phase of antigen presentation by S^- cells (day 0 or day 1 of culture; Table 2) is consistent with this idea. Under conditions where the antigen-presenting cells have intrinsic stimulating activity, CSCS acts as an amplifier of the cytotoxic cell response. This amplification of the intrinsic response was observed when CSCS was added to cultures as late as the third day of culture. At this stage, we cannot say whether the costimulator and amplifier factors are the same or different molecules present in the CSCS preparation.

CSCS preparations produced from C57Bl/6, CBA, or C3H spleens show essentially the same activating effect on the response of C57Bl/6 lymph node cells to antigen carried by S^- cells (Table 3). Thus, the nonspecific signal is not strain specific. The nonspecific signal does, however, show a pronounced species specificity and significant responses of bovine and human lymphocytes to P815 were only obtained in the presence of homologous factor (Table 4). Neither these factors nor a factor obtained from guinea pig spleen cells had any effect on the response of mouse lymphocytes to UV-irradiated P815 cells. However, the mouse factor did have some effect on the response of guinea pig lymphocytes to the mouse tumor. These findings substantiate our earlier prediction that the species specificity of allogeneic activity is expressed at the level of the second or nonspecific signal (3).

The activity of CSCS in these experiments cannot be due to contamination with Con A. All of the Con A that was not cell bound was removed from the spleen cultures by three washes after the initial 2-hr culture. Con A could not be detected in the final CSCS by erythrocyte agglutination, nor could activity be removed from CSCS on a Sephadex G-10 column (data not shown). In addition, Con A added to mixed cell cultures over a wide range of concentrations did not reproduce the effect of CSCS. Perhaps the most convincing evidence that the activity of CSCS is not due to Con A is its species specificity (Table 4).

CSCS may be identical to several other spleen cell factors that have been prepared in a similar way. Many of these, e.g., T cell-replacing factor (17) and nonspecific macrophage factor (18), are detected by their activity on B cells. Another, lymphocyte-activating factor (19), enhances the nonspecific proliferative response of thymocytes to mitogens. CSCS also resembles mitogenic factor (20) made by incubating immune guinea pig lymphocytes with antigen in serum-free medium.

This paper provides a demonstration of a nonspecific factor that converts a nonstimulating cell to one that induces a full and specific cytotoxic response. CSCS is clearly not a pure substance because it is made by a procedure reported to be excellent for preparing macrophage migration inhibitory factor. Its separation, characterization, and mechanism of action are suitable questions for subsequent research.

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