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Antigen-presenting cell function in induction of helper T cells for cytotoxic T-lymphocyte responses: Evidence for antigen processing

(macrophages/liposomes)

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ABSTRACT We demonstrate that splenic adherent cells (SACs) play an active role in the presentation of H-2K^k antigen for an alloreactive cytotoxic T-lymphocyte (CTL) response. If antigen is incubated with SACs for 12 hr, they will provide maximal stimulation and present the antigen in the context of their Ia molecules. UV irradiation of these SACs, prior to the 12-hr incubation with H-2K^k antigen, abrogates this stimulatory capacity. Macrophage-bound antigen is not sufficient for stimulation of a response; a second signal is required as well, that, in our system, is provided by phorbol myristic acetate. The SACs are involved in the activation of helper T cells; however, they are not required for presentation of antigen to the precytotoxic T-lymphocyte, which requires two signals for activation, one provided by antigen and the other by a T-cell-derived helper factor.

Mononuclear phagocytes are a crucial component of immune responses in humoral (1, 2), proliferative (3, 4), and cytotoxic systems (5–7). Macrophages are required for the uptake and presentation of soluble protein antigens that precedes *in vitro* T-lymphocyte proliferation and T- and B-lymphocyte collaboration for an antibody response. Further, macrophage-lymphocyte interactions are regulated by the major histocompatibility complex (8–11), specifically, by the Ia molecules on the sensitizing macrophages (12–14). Macrophage uptake and presentation is not limited to soluble protein antigens; it has recently been shown that radio-resistant splenic adherent cells (SACs) are required for the generation of cytotoxic T-lymphocyte (CTL) responses to both allogeneic membrane proteins (7) and trinitrophenyl-modified cells (6).

It is possible to isolate MHC antigens that are serologically active and able to stimulate a CTL response. The H-2K^k antigen can be purified from cell membranes by affinity chromatography with a monoclonal anti-H-2K^k antibody column (15), and this antigen has been shown to stimulate an allogeneic response when incorporated into liposomes (16). Using this antigen, we have shown that Ia$^+$ SACs are involved in the presentation of H-2K^k and that the purified alloantigens appear to be presented in the context of the self-Ia of the SACs (7). We have also shown that the Ia$^+$ SACs present antigen to Ly 1$^+$ helper T cells that are then triggered to elaborate a soluble helper factor (T_HF). This factor can serve as one of the signals required for prekiller cell activation (12).

Although the requirement for macrophages in the generation of CTL responses is established, the nature of their actual function has not yet been clearly defined. It is currently believed that *in vitro* helper T-cell activation requires at least two signals: one provided by antigen and one provided by an accessory cell. Several macrophage-derived soluble factors that potentiate T-cell responses to mitogens (17, 18) and alloantigens (19) have been described. It has been speculated that such a factor, termed lymphocyte-activating factor (LAF) (17) or interleukin 1 (see ref. 20 for revision to the nomenclature), provides the "second signal" required for T-cell activation. Rosenstreich *et al.* (21) have shown that a synthetic compound, phorbol myristic acetate (PMA), can replace macrophages as a source of this activating signal. The availability of such a reagent is a useful tool for separating the components of macrophage function.

In these studies, we examined the nature of the antigen signal provided to the helper T cell. We took advantage of the fact that UV irradiation interferes with the antigen-presenting function of macrophages (22, 23). By replacing the second signal with PMA, we demonstrated that induction of the helper T-cell pathway for an alloreactive CTL response requires interaction of macrophages with the antigen before UV irradiation.

MATERIALS AND METHODS

Mice. (BALB/c × DBA/2)F_1 (CD2F_1) (*H-2^d*) mice 6–12 weeks of age, were purchased from Cumberland Farms (Clinton, TN).

Antisera. The antisera used were generously provided by M. Dorf. The reagents and their cytolytic titers were as follows: anti-Ia^d, (C3H × LG/ckc)F_1 anti-C3H.OH, 1:512, anti-Ia^s, (CP × A.TL)F_1 anti-A.TH, 1:512; anti-D^d, (B10 × LP.RIII)F_1 anti-B10.A(18R), 1:1024; and anti-K^k, (B10 × A.TL) anti-4R, 1:128.

Liposome Preparation. H-2K^k protein was purified from RDM-4 tumor cells by using a monoclonal antibody affinity column as described (15). Liposomes were prepared in 0.5% deoxycholate: detergent-insoluble membrane proteins from P815 cells, lipid from P815 cells, H-2K^k protein, and a trace of ^{125}I-labeled H-2K^k. This mixture was dialyzed against Tris-buffered saline and 5 mM $CaCl_2$, and the liposomes were then harvested by centrifugation for 45 min at 100,000 × *g*. These procedures have been detailed (7).

Reagents. The T helper factor was generously provided by R. Gallo and J. Mier of the National Cancer Institute. It was derived from the supernatant of PHA-stimulated human lymphocytes. The precipitate after 50% saturation with $(NH_4)_2SO_4$, was discarded; then, the precipitate at 75% saturation was dissolved and dialyzed and added to cultures at a final dilution of 1:10 (24). PMA was obtained from Sigma and was added to cultures at 10 ng/ml.

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Abbreviations: SAC, splenic adherent cell; CTL, cytotoxic T lymphocyte; PMA, phorbol myristic acetate; T_HF, T helper factor; CD2F_1, (BALB/c × DBA/2)F_1; LAF, lymphocyte-activating factor.

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Preparation of SACs. This procedure has been described (7, 25). Briefly, 5×10^7 spleen cells were incubated in 100-mm glass petri dishes for 2–3 hr at 37°C. Nonadherent cells were removed by gentle agitation and several washes. Adherent cells were detached by incubation at 37°C with 5 ml of a 1:5000 dilution of Versene buffer (GIBCO) for 15 min. The cells were then incubated overnight at 37°C on a roller drum with or without antigen. When the SACs were pulsed with antigen, 10–15 $\times 10^6$ cells were incubated with ≈ 2–4×10^7 cell equivalents of ^{125}I-labeled H-2K^k antigen (10–60,000 cpm) incorporated into liposomes. After the appropriate incubation, the SACs were extensively washed and the number of cell equivalents of antigen bound was determined by the amount of ^{125}I bound.

UV Irradiation. UV light irradiation was by a bank of six FS-40 "Sun Lamp" fluorescent tubes (Westinghouse, Pittsburgh, PA). These tubes provide a continuous UV spectrum that has a peak at 313 nm. Tube-to-target distance was 20 cm, and UV output as measured by I. L. 443 Phototherapy Radiometer (International Light, Inc., Newburyport, MA) was 1.1–1.4 (mJ/cm^2)/sec. Cells in medium were exposed to UV irradiation for 2 min in quartz cuvettes. UV irradiation did not significantly affect uptake of ^{125}I counts.

Depletion of SACs. Spleen cells were depleted of SACs by two cycles of adherence to plastic dishes (Falcon #3003 tissue culture dishes) followed by passage over nylon wool, as described (7).

Generation of CTL and ^{51}Cr Release Assay. CD2F$_1$ mice were primed intraperitoneally with YAC(H-2^a) tumor cells. Spleen cells were removed 4–12 weeks after immunization, and 7×10^6 cells were cultured with the indicated antigen in 16-mm Linbro wells.

After 5 days, cells were harvested and assayed in a 4-hr ^{51}Cr release assay on the indicated target cells. Data were calculated as percent specific release (7).

RESULTS

That SACs are required for the induction of a CTL response to purified alloantigens has been shown (7). To determine the nature of the SACs required for antigen presentation, we measured the stimulatory capacity of UV-irradiated SACs in the presence of a synthetic substitute for macrophage-derived factors. SACs from nonprimed CD2F$_1$ mice were pulsed with antigen (H-2K^k in liposomes) for 48 hr and then washed extensively. The SACs were UV irradiated either before or after the 48-hr pulsing. These SACs were then cocultured with syngeneic nylon-wool-purified responder cells that had been primed *in vivo* to YAC(H-2^a) tumor cells. We found that UV irradiation of the SACs, either before or after they were pulsed with antigen, effectively abrogated any response (Table 1). The addition of PMA to the cultures, which has been shown to substitute for the macrophage signal, LAF (or interleukin 1) (21), reconstituted the response only when the SACs had been treated with UV light after the exposure to antigen. PMA alone was not stimulatory.

The kinetics of the interaction of the SACs with antigen were studied. We found that more than 4 hr of interaction were required when the antigen pulsing preceded the UV-irradiation of the SACs and the PMA was added to the cultures (Fig. 1) and that, even after 12 hr, the response induced was not maximal.

We had previously shown that Ia$^+$ SACs are required to present H-2K^k antigen and felt that Ia might be simply a marker for a subset of SACs that produce LAF or interleukin 1. Thus, we wanted to determine whether the Ia molecules were an obligatory component of antigen presentation to the helper cell. Splenic adherent cells were pulsed with H-2K^k for 48 hr. The unbound antigen was then washed away, and the SACs were UV irradiated. The pulsed SACs were cocultured with primed CD2F$_1$ responder cells that had been depleted of SACs. The addition of PMA to the cultures reversed the effect of UV irradiation, but stimulation by the SACs was still blocked by the addition of the appropriate anti-Ia antiserum to the culture

Table 1. UV irradiation of SACs prevents antigen presentation

Responder	Stimulator	UV Irradiation	Lysis of RDM-4 60:1	Lysis of RDM-4 30:1
CD2F$_1$	—		20	5
CD2F$_1$ + PMA	—		13	4
CD2F$_1$	H-2K^k pulsed on SACs	—	74	44
CD2F$_1$	H-2K^k pulsed on SACs	Before pulsing	28	4
CD2F$_1$ + PMA	H-2K^k pulsed on SACs	Before pulsing	19	6
CD2F$_1$	H-2K^k pulsed on SACs	After pulsing	21	11
CD2F$_1$ + PMA	H-2K^k pulsed on SACs	After pulsing	61	49

Spleen cells from (BALB/c × DBA/2)F$_1$ mice primed *in vivo* to YAC(H-2^a) were depleted of SACs and then cultured for 5 days with UV-irradiated or normal syngeneic SACs bearing 5×10^4 cell equivalents of H-2K^k antigen. Where indicated, PMA was added at a concentration of 10 ng/ml. Values are percent specific release of ^{51}Cr at the indicated effector-to-target ratios. Spontaneous release of RDM-4 in all experiments was <13%.

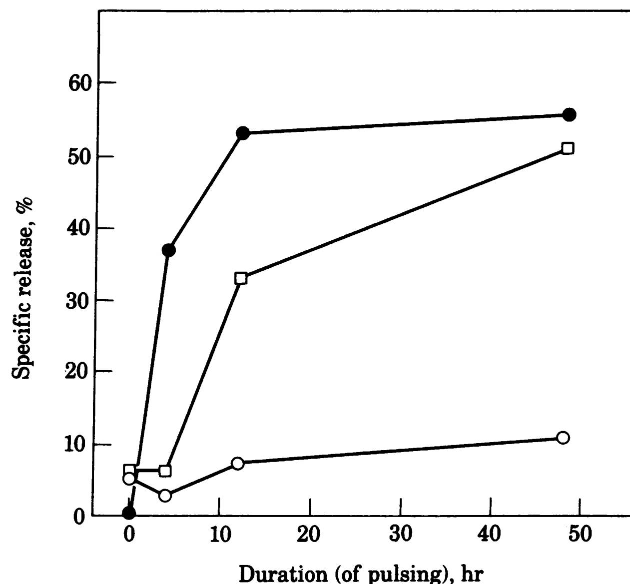

FIG. 1. Kinetics of interaction of SACs with antigen. Spleen cells from CD2F$_1$ mice primed *in vivo* to YAC were depleted of SACs and cultured for 5 days with syngeneic SACs bearing 5×10^4 cell equivalents of H-2K^k antigen. The SACs were pulsed with antigen at 37°C for the indicated time period and then UV irradiated. All SACs were incubated for a total of 48 hr before being added to cultures containing primed T cells. For example, SACs that were UV irradiated after 12 hr with antigen were then incubated for a further 36 hr before their addition to cultures containing primed T cells. CTL activity was assayed on ^{51}Cr RDM-4 tumor targets at an effector-to-target ratio of 60:1. ●, SACs pulsed with H-2K^k; ○, SACs pulsed with H-2K^k and irradiated; □, SACs pulsed with H-2K^k, irradiated, and added to culture with PMA (10 ng/ml).

Table 2. Ia antigen of the SACs is involved in antigen presentation even after UV irradiation

Responder	Stimulator	UV irradiation	Antiserum*	Lysis of RDM-4		
				100:1	33:1	50:1
		Experiment 1				
$CD2F_1$	—			24	7	
$CD2F_1$ + PMA	—			18	11	
$CD2F_1$	$H\text{-}2K^k$ pulsed on SACs	—		63	45	
$CD2F_1$	$H\text{-}2K^k$ pulsed on SACs	—	αIa^d	20	9	
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	—	αIa^d	12	5	
$CD2F_1$	$H\text{-}2K^k$ pulsed on SACs	After pulsing		27	16	
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	After pulsing		56	37	
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	After pulsing	αIa^d	16	7	
		Experiment 2				
$CD2F_1$ + PMA	—					−2
$CD2F_1$ + T_HF	—					7
$CD2F_1$	$H\text{-}2K^k$ pulsed on SACs	—				56
$CD2F_1$	$H\text{-}2K^k$ pulsed on SACs	Before pulsing				5
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	Before pulsing				7
$CD2F_1$ + T_HF	$H\text{-}2K^k$ pulsed on SACs	Before pulsing				49
$CD2F_1$	$H\text{-}2K^k$ pulsed on SACs	After pulsing				11
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	After pulsing				51
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	After pulsing	αIa^d			15
$CD2F_1$ + PMA + T_HF	$H\text{-}2K^k$ pulsed on SACs	After pulsing	αIa^d			44
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	After pulsing	αIa^s			48
$CD2F_1$ + PMA	$H\text{-}2K^k$ pulsed on SACs	After pulsing	αD^d			45

Spleen cells from (BALB/c × DBA/2)F_1 mice primed to YAC were cultured for 5 days with SACs that had been pulsed with liposomes containing $H\text{-}2K^k$ antigens (experiment 1, 5×10^4 cell equivalents; experiment 2, 3×10^4 cell equivalents). Where indicated, PMA was added at a final concentration of 10 ng/ml and T_HF was added at a final dilution of 1:10.

* Added to a final concentration of 1% at the initiation of cultures.

(Table 2). Similar results were obtained by using rat monoclonal antibodies directed to I-region gene products (data not shown). It has previously been shown that the addition of a T-cell-derived helper factor (T_HF) to CTL cultures can bypass the requirement for any component of the helper pathway, including the accessory cell. The addition of T_HF to cultures bypassed the blocking by anti-Ia antiserum and also allowed a response to develop with SACs that had been UV-irradiated before antigen pulsing.

DISCUSSION

Helper T cells recognize antigen much more efficiently when it is presented bound to SACs rather than added directly to culture (7, 26), even in the case of integral membrane proteins such as H-2 (7). There has been controversy concerning the exact role played by these SACs—specifically, whether the capacity of the macrophage to present antigen is a function of macrophage-induced alterations of the antigen (i.e., antigen processing). Thomas has argued against antigen processing, based on the observation that glutaraldehyde treatment of macrophages before trinitrophenyl modification does not abrogate stimulation of T-cell proliferation (27). Korngold and Sprent, however, have argued for antigen-processing, based on studies on the specificity of the killers induced by glutaraldehyde-treated cells (28), and Bevan has suggested that the crosspriming that he observed *in vivo* is due to representation of the antigen by host macrophages (29). It is known that antigen is ingested by the mononuclear phagocytes and undergoes varying degrees of catabolism, but not all the antigen appears to be degraded; a small proportion can be detected on the macrophage surface essentially in its native configuration (26). There have been conflicting reports on the ability to block antigen presentation with antibodies to the native antigen (4, 30, 31), and these studies are subject to the limitations of the serological reagents used (e.g., their specificities and steric properties).

We have demonstrated that stimulation of an alloreactive CTL response requires that the accessory cells interact with the antigen before UV irradiation. This suggests that the SACs play an active role in the presentation of antigen. Our data indicate that UV treatment abrogates any stimulatory capacity of the SACs and that PMA reconstitutes the response only in the presence of SACs that have been functional for more than 4 hr in the presence of antigen. Thus, macrophage-bound antigen is necessary but not sufficient for stimulation of a response; a factor, most probably derived from the same accessory cells, is also required. Furthermore, even in the presence of PMA, anti-Ia antiserum specific for the Ia determinants on the SACs, blocks induction of a response. We conclude that, in this system, there are at least two distinct components to accessory cell function, neither of which can be accomplished by a UV-irradiated cell: (*i*) the elaboration of a lymphocyte-activating factor and (*ii*) the presentation of antigen in the context of the Ia of the accessory cell, possibly following some sort of macrophage-induced modification. It is possible (*i*) that antigen is internalized and then modified and re-expressed, (*ii*) that it must be re-expressed by the macrophage to be recognized in the proper configuration with Ia, or (*iii*) that proteolytic enzymes convert it to an immunogenic form extracellularly.

The kinetic studies show that, if the SACs are not disrupted, pulsing with antigen for 4 hr is sufficient to allow almost maximal stimulation. Although the SACs were washed after 4 hr, they probably continue to internalize and digest antigen that is bound to the cell surface. If, however, the SACs are UV-irradiated after the antigen pulsing, PMA will reconstitute the response only if the SACs have been allowed to interact with the antigen for 12 hr.†

† It has not been determined whether the PMA is substituting for a macrophage-derived factor and directly affecting the T cells or is activating the residual macrophages in the responder population and inducing them to produce more LAF.

Korngold and Sprent (28) have shown that, for minor histocompatibility antigens, glutaraldehyde treatment of the immunizing spleen cells prevents stimulation by H-2 incompatible cells and have suggested that the treated cells could not be effectively processed by host macrophages and thus were not recognized in the context of syngeneic Ia. Our data suggest that the antigen, $H\text{-}2K^k$, must be presented in the context of the Ia determinants of the responder cells after uptake by functional SACs. This presentation of antigen by macrophages seems to be required for the induction of helper cells but not of pre-killers. We have found that T_HF can substitute for both the SACs and the helper cells required for a CTL response to $H\text{-}2K^k$ (12). We have also found that anti-Ia^d antiserum directed against the I-region antigens of the SAC population inhibits the CTL response. If, however, the helper cell requirement is bypassed by addition of T_HF, the presence of anti-Ia^d antiserum in the culture has no effect on the response. Evidence that the pre-CTL can recognize alloantigen bound to UV-inactivated SACs was provided by experiments using SACs that were UV-irradiated before antigen pulsing. Antigen pulsed onto UV-irradiated SACs did not stimulate a response. When T_HF was added, however, a good response was obtained, showing that the antigen signal was available to the pre-CTL.

In conclusion, we have evaluated, the requirements for the triggering of the helper T cell and the pre-CTL. Helper T-cell activation requires antigen that has interacted with SACs before UV irradiation, and this antigen must be seen by the helper T cell in association with the Ia of the SAC. A second signal provided by the SAC, replaced by PMA, is also necessary. Pre-CTL triggering requires antigen, but it is not required that the antigen be presented in the context of the Ia of the SAC. The pre-CTL also requires a second signal, in this case, provided by the helper T cell, and this signal can be replaced by the addition of T_HF.

The approach described here provides us with the ability to analyze how the SACs function as antigen-presenting cells and how they "process" a well-defined antigen, $H\text{-}2K^k$. We can now study on a molecular level whether antigen modification is required for helper-cell activation and whether the helper T cell and the prekiller cell recognize the same form of antigen, as well the role played by the Ia antigens of the presenting cell.

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