Brief Definitive Report

CONCOMITANT INDUCTION OF THE CELL SURFACE EXPRESSION OF Ia DETERMINANTS AND ACCESSORY CELL FUNCTION BY A MURINE MACROPHAGE TUMOR CELL LINE*

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One of the most important aspects of the experimental analysis of the mechanism of antigen presentation is the development of a defined, in vitro culture system in which one can analyze the interaction of monoclonal populations of accessory cells and major histocompatibility complex (MHC)-restricted, antigen-dependent populations of responder lymphocytes. Recent technical advances (1, 2) in cell culture and hybridization procedures have now provided the capacity to establish defined populations of MHC-restricted, antigen-specific responder lymphocytes for in vitro analysis. Recent studies (3-5) have clearly demonstrated the ability to induce cell surface Ia expression on various populations of normal murine macrophage. However, there are no good examples of homogeneous, continuous, Ia-positive, macrophage cell populations that can be used as potential accessory cells in such a defined in vitro system.

We describe the induction of cell surface Ia molecules on an Ia-negative, murine macrophage tumor, WEHI-3, and the subsequent ability of this Ia-induced tumor cell line to provide accessory cell function in an MHC-restricted, antigen-driven, in vitro assay system. The assay system used measures the ability of various accessory cell populations to stimulate a series of MHC-restricted, antigen-specific T-T hybrids to synthesize and secrete IL-2 (2).[1]

Materials and Methods

Antibodies. The H-$2D^d$ and H-$2K^d$ alloantisera were generated from the strain combinations (B10.AKM × $129)F_1$ anti-B10.A and (B10.A(2R) × $A.CA)F_1$ anti-HTG, respectively, and were obtained from Dr. John Ray of the National Institute of Allergy and Infectious Diseases. The A.TH anti-A.TL alloantisera used in the blocking experiments was provided by Dr. C. Spellman, Univ. of New Mexico School of Medicine, Albuquerque, NM. Monoclonal antibodies against the Ia.15 (I-A^d) and Ia.7 (I-E/C^d) specificities used in the Ia blocking experiment were provided by Dr. David McKean of the Mayo Clinic, NY. MKD6 monoclonal antibody, generously provided by Dr. John Kappler and Dr. Philippa Marrack, National Jewish Hospital and Research Center, Denver CO., recognizes an as yet undefined specificity on the I-A^d molecule (2). The MAC-1 monoclonal antibody was provided by Dr. T. Springer, Harvard

* Supported by grant CA-22105 from the National Institutes of Health.

[1] Walker, E. B., N. L. Warner, R. Chestnut, H. Grey, J. W. Kappler, and P. Marrack. 1981. Antigen-specific, MHC-restricted interactions in vitro between tumor cell lines and T cell hybridomas. Manuscript submitted for publication.

University, Cambridge, MA. The FITC-labeled second step reagents, fluorescein isothiocyanate (FITC) goat anti-mouse $\gamma 2$, and FITC goat anti-mouse $\gamma 1$ sera were obtained from Meloy Labs, Springfield, VI.

Immunofluorescence and Flow Cytometry (FCM). The techniques of immunofluorescence staining, FCM and analysis, and FCM data analysis have been described in detail previously (6). Immunofluorescence was measured by FCM analysis using the Los Alamos National Laboratories flow system.

Cell Lines. The murine myelomonocytic leukemia, WEHI-3, has been carried in suspension culture in our laboratory for several years and was originally developed in the BALB/c mouse at the Walter and Eliza Hall Research Institute, Melbourne, Australia (7). The IL-2-sensitive indicator cell, HT-2, has been described previously (8). The origin, MHC restriction, and antigen specificity of the two T-T hybridomas used as responder cells in this study have been described in detail elsewhere (2, 9).[1] Briefly, AODK-3.4 produces IL-2 only in the presence of ovalbumin (OVA) and an accessory cell that is I-A^k positive; AODH-7.1 responds to human gamma globulin (HGG) plus an accessory cell that is positive for the I-E^d cistron product. All the T-T hybridoma cell lines were provided by Dr. J. Kappler and Dr. P. Marrack.

Accessory Cell Function Assay System. The assay system used to measure accessory cell function has been described in great detail elsewhere (2).[1] Briefly, the assay consists of placing 2×10^5 T-T hybrid cells together with various tests populations of accessory cells, with or without antigen. The T-T hybrid cells will generate IL-2 only in the combined presence of a syngeneic accessory cell and the appropriate specific antigen. After 24 h, the culture supernatants of each test well were titrated by twofold serial dilution to a final dilution of 1/64. An IL-2-sensitive indicator cell, HT-2, was added to each well, and proliferation was assayed visually or by thymidine incorporation.

Ia Induction. The supernatant used to induce cell surface Ia on WEHI-3 was generated by treating rat spleen cell suspension cultures with a 48-h pulse of concanavalin A (Con A), as previously described (10). This initial Con A-induced supernatant was diluted to a 50% concentration in Dulbecco's minimum essential medium (DMEM) (buffered for 12% CO_2) + 10% fetal calf serum (FCS) (referred to as rat Con A S/N). WEHI-3 was seeded in this media at a concentration of 1×10^5 cells/ml to 2×10^5 cells/ml and incubated for 48 h at 37°C. Subsequently the cells were washed three times in sterile basic salt solution and recultured at 2×10^5 cells/ml in factor-free DMEM 10% FCS for 16-24 h. Cells were washed once and used in the T-T hybrid assay.

Results and Discussion

FCM Analysis of Ia-induced WEHI-3. Using indirect immunofluorescence and quantitative flow cytometry analysis, we examined WEHI-3 cells for cell surface expression of MHC gene products. As shown in Fig. 1, WEHI-3 tumor cells showed minimum reactivity with the anti-Ia antibodies (panel A, B), but did react significantly with antibodies directed against H-$2D^d$ and H-$2K^d$. In contrast, incubation of WEHI-3 in the presence of rat Con A S/N for 48 h induced cell surface Ia determinants, with >90% of the cell population showing positive staining for the I-A and the I-E/C gene products (panels E, F). As shown in panels G and H, the amount of cell surface H-$2K^d$ and H-$2D^d$ determinants is also significantly increased. Quantitative comparison of the induced vs. uninduced cells indicated an ~3.3-fold increase in surface H-$2K^d$ determinants and a 2.5-fold increase in H-$2D^d$ determinants. In contrast to the results obtained with rat Con A S/N, appropriate control supernatants from various Con A-induced, murine T cell tumor preparations consistently failed to elicit Ia induction on WEHI-3. Continual presence of the "Ia inducing factor" (IaIF) is required to maintain the Ia-positive phenotype; the induced Ia expression is significantly reduced on tumor cells incubated in factor-free media for periods >24 h. More detailed kinetic studies of Ia expression are being pursued using quantitative FCM analysis and metabolic labeling of Ia determinants. Preliminary experiments indicated that P388.DI and

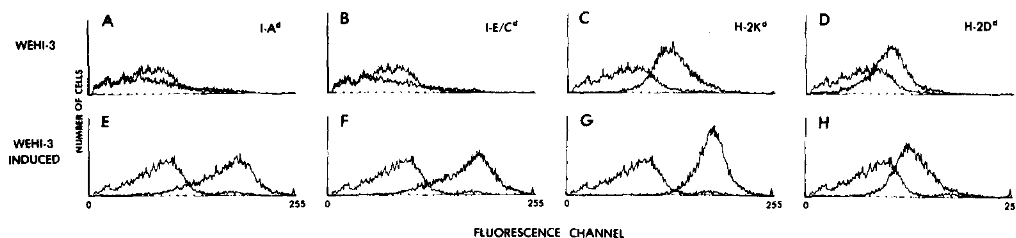

FIG. 1. Induction of I region determinants on WEHI-3. WEHI-3 cells were cultured in the absence (panels A–D) or presence (panels E–H) of rat Con A S/N for 48 h, washed extensively, and examined for the presence of MHC determinants. Cells were incubated with predetermined optimum amounts of anti-I-A^d (MKD6) M.Ab. (panels A, E), anti-I-E/C (Ia.7) M.Ab. (panels B, F), anti-H2K^d serum (panels C, G), and anti-H-2D^d serum (panels D, H), followed by incubation with FITC goat anti-mouse $\gamma 2$ serum. 10,000 cells were analyzed using the Los Alamos National Laboratories flow system. In each figure, the fluorescence histogram of the stained sample is superimposed over the fluorescence histogram of the NMS control (left peak). Voltage and gain settings on the fluorescence amplifier were identical for all samples (800 v, gain 2.0). The X axis indicates 255 channels, 3 decade log scale, increasing fluorescence intensity left to right; Y axis, number of cells. Note induction of Ia determinants and increased expression of H-2 determinants on WEHI-3 cells cultured in rat Con A S/N. Specificity controls indicated that induced WEHI-3 cells did not react with antibodies against MHC specificities of inappropriate haplotypes, such as H-2.2 or Ia.2 (not shown). MKD6, Ia.7, and Ia.2 M.Ab. are all $\gamma 2$ antibodies.

WEHI-265 (Abelson virus-induced myelomonocytic leukemia) are also capable of expressing Ia, using this induction procedure. In contrast, certain other lines, such as PU5.1R, WEHI-274, and certain sublines of WEHI-3, did not respond (data not shown). Further studies with these tumors are in progress.

Coulter Volume to Fluorescence Intensity Analysis. After treating the WEHI-3 macrophage tumor cell line with rat Con A S/N, it was noted that the cell line seemed to go into proliferative arrest; the doubling time increased from 16 to 48 h, and the cells become visably larger by microscopic examination and coulter volume determination. Therefore, it was of concern to see whether the increase in Ia-positive cells was simply related to an overall increase in cell volume upon treatment with the factor source. Thus, it was possible that induced cells might express more total Ia antigen per cell because of a greater cell surface area, and, as a consequence, might be detected as Ia positive, whereas the smaller uninduced cells might lack sufficient total Ia antigen to be detected. To test this possibility, the relationship of cell volume of fluorescence intensity was determined, using the flow systems of the Los Alamos National Laboratories. This type of analysis has been described in detail elsewhere (4). Fig. 2 shows the mean fluorescence, in volts, plotted vs. the coulter channel fraction. Panels A and B clearly show that there is a dramatic increase in I-A, I-E/C, and H-2K^d determinants on induced WEHI-3 cells, compared with uninduced controls for cells in all the coulter channel fractions. Panels C and D show that there is a smaller but significant increase in H-2D^d determinants and surface antigens recognized by a macrophage-specific monoclonal antibody, MAC-1, in all coulter channel fractions. Thus, increase in cell surface MHC determinants, as measured by flow cytometry, is not the result of simple increases in cell surface, but indicates increased surface density of these specificities. Similar analysis using monoclonal antibodies against other non-MHC-related determinants, such as Ly-5, showed no significant cell surface change in density for these markers (unpublished data).

Because there was a profound, proliferative arrest observed concomitant with the Ia induction phenomenon, we analyzed the DNA profile of IaIF-treated WEHI-3

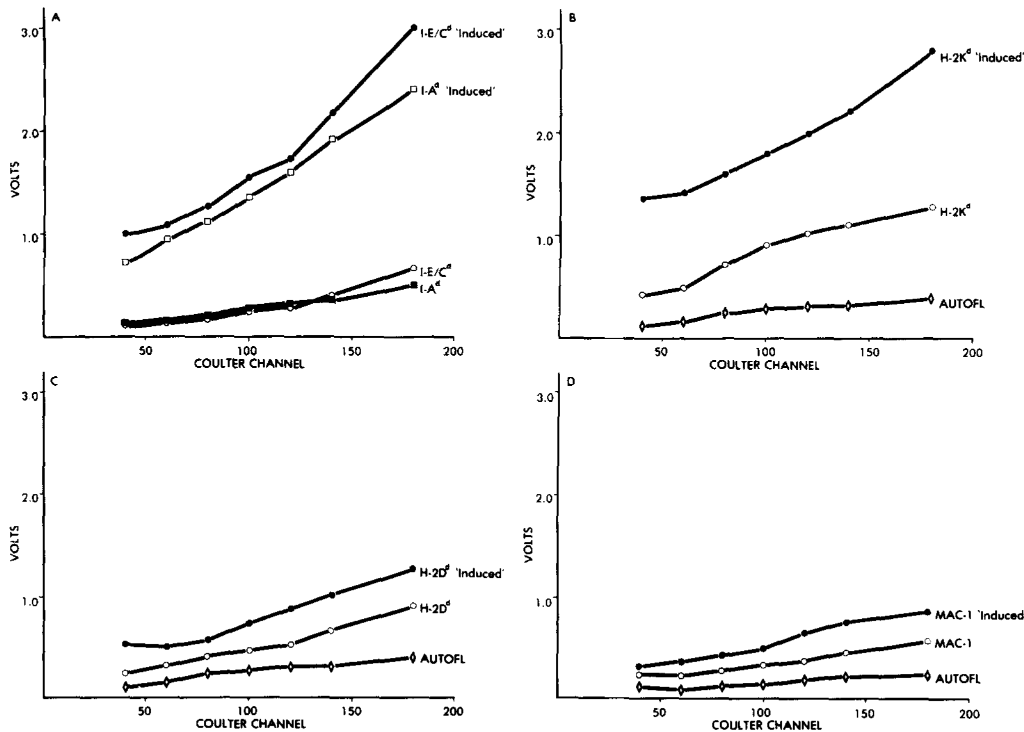

FIG. 2. Relationship of fluorescence intensity to coulter volume on WEHI-3 cells. WEHI-3 cells, cultured in the absence or presence of rat Con A S/N, were stained with NMS (autofluorescence control), anti-I-A^d (MKD6) M.Ab., anti-I-E/C^d (Ia-7) M.Ab., anti-H-$2K^d$ serum, and anti-H-$2D^d$ serum, followed by FITC goat anti-mouse $\gamma 2$ serum, and anti-MAC-1 M.Ab., followed by FITC mouse anti-rat kappa M.Ab. Correlated measurement of surface fluorescence and coulter volume were determined using the Los Alamos National Laboratories flow systems. The coulter distribution of each sample was divided into 20 channel increments, and the mean fluorescence intensity of cells falling within each 20 gated channel was determined and expressed as mean volts. In this figure, the mean fluorescence vs. coulter channel is displayed for each sample. In panel A, the autofluorescence of the uninduced WEHI-3 cells (not shown) totally overlapped with the anti-Ia stained samples. Note the significant increase in MHC determinant expression on the induced WEHI-3 cells in all coulter channel fractions, indicating increased density of surface antigen.

cells, using mithramycin staining and flow cytometry analysis, as described previously (6). These studies show that there is a significant loss of cells in the S phase and accumulation of cells in G1 and G2/M. Preliminary experiments using fractions of isoelectric focusing gel separation of rat Con A S/N indicate that the activity responsible for Ia induction is not the activity causing the proliferation arrest (unpublished data).

Accessory Cell Function Analysis. The two representative experiments shown in Table I clearly demonstrate that Ia-induced WEHI-3 activates AODH-7.1 to produce IL-2 in an antigen-dependent fashion, although it does not stimulate the I-A^k-specific cell line AODK 3.4. Further, uninduced WEHI-3 has little or no effect in triggering IL-2 production from AODH-7.1. Approximately 60% of the IL-2 response of AODH-7.1 can be blocked by treating WEHI-3 with anti-Ia.7 monoclonal antibody; the anti-Ia.15 monoclonal reagent has virtually no effect. Blocking the WEHI-3 tumor line with A.TH anti-A.TL completely abrogates the IL-2 production by AODH-7.1, whereas NMS has no effect on the response. We are also presently engaged in

TABLE I

Analysis of the Ability of Uninduced and Ia-induced WEHI-3 Cells to Stimulate IL-2 Production by MHC-restricted, Antigen-dependent, T-T Hybridomas

Experiment 1	[^{3}H]TdR (cpm)			
	(OVA)	AODK-3.4	(HGG)	AODH-7.1
B10.A spleen	+	32,096 (1/16)		
	−	1,200		
BALB/c spleen			+	73,998 (1/64)
			−	1,012
Media control	+	900	+	1,286
WEHI-3 (uninduced)	+	1,400	+	1,600
			−	1,480
WEHI-3 (induced)	+	2,820	+	37,896 (1/16)
			−	3,640
WEHI-3 (induced) + Ia.7 M.Ab			+	16,168 (1/8)
WEHI-3 (induced) + Ia.15 M.Ab			+	38,620 (1/16)
Experiment 2				
B10.A spleen	+	28,640 (1/16)		
	−	4,896		
BALB/c spleen			+	62,646 (1/64)
			−	6,720
Media control	+	5,265	+	5,812
WEHI-3 (uninduced)	+	5,716	+	5,314
			−	4,581
WEHI-3 (induced)	+	5,566	+	37,893 (1/16)
			−	6,201
WEHI-3 (induced) + NMS			+	36,468 (1/16)
WEHI-3 (induced) + A.TH > A.TL			+	7,211

Microculture wells were prepared with 2×10^5 hybridoma cells and 200 μg of the appropriate antigen in 200 μl of culture media. Either 10^6 irradiated (4,000 rad) spleen cells, 10^5 tumor cells, or media alone (control) was added to provide accessory cell function. Results indicate the amount of radiolabeled thymidine incorporation by 5,000 HT-2 cells incubated in the undiluted supernatant from the test culture and are an indicator of the amount of IL-2 generated. The number in parenthesis is the highest dilution at which the test supernatant demonstrates IL-2 activity >25% above the media control. Anti-Ia blocking was carried out by treating 5×10^5 WEHI-3 cells with 25 μl of the optimum dilutions of monoclonal reagents and A.TH anti-A.TL.

experiments to test the ability of several of the Ia-induced macrophage tumors to provide accessory cell function in stimulating other T-T hybridomas that recognize specific antigen only in the context of an I-A^d gene product.

The data presented in this preliminary study clearly demonstrate that selected murine tumors of the monocyte-macrophage lineage can be induced to express serologically defined, cell surface Ia determinants. The expression of these determinants is paralleled by the ability of the Ia-induced macrophage tumor line to provide accessory cell function in at least one type of MHC-restricted, antigen-dependent in vitro assay system. Further, the accessory cell function can be partially or totally blocked by various anti-Ia antibodies. Although these observations do not prove any necessary causal relationships between the appearance of cell surface Ia and accessory cell function, they are consistent with the notion that Ia molecules play a central role in antigen processing and presentation. This study may provide the first opportunity to initiate the analysis of the mechanism of accessory cell function, using defined, homogeneous populations of tumor cells of the monocyte-macrophage compartment as antigen-presenting cells.

Summary

This study demonstrates that an uncharacterized soluble factor produced in concanavalin A-induced rat spleen cell suspensions has the capacity to induce the

increased expression of cell surface H-2K and H-2D molecules and the expression of I-region gene products on murine monocyte-macrophage lineage tumors that are not Ia positive in the absence of the factor. In parallel with induction of serologically defined Ia specificities, Ia-induced WEHI-3 macrophage tumor cells are capable of providing accessory cell function in stimulating IL-2 production by T-T hybridomas that are activated in a major histocompatibility complex-restricted, antigen-dependent fashion. The uninduced Ia-negative WEHI-3 tumor cells do not trigger a comparable response in this assay system.

Received for publication 1 October 1981 and in revised form 18 November 1981.

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