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Rat dendritic cells function as accessory cells and control the production of a soluble factor required for mitogenic responses of T lymphocytes

(macrophage/periodate/neuraminidase plus galactose oxidase/interleukin/indirect stimulation)

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ABSTRACT **Transformation of T lymphocytes, induced by treatment with periodate or with neuraminidase plus galactose oxidase, requires the participation of accessory cells. Procedures were developed for the fractionation of rat lymph node cells, by which most of the lymphocytes can be recovered as a major population of cells that do not respond to mitogenic stimulation unless accessory cells from a separated minor population are added. Further purification led to a 1000-fold overall increase in accessory activity per cell, with a 50–70% yield. The purest preparations were virtually free of macrophages and contained more than 90% typical dendritic cells. Maximum responses occurred at a ratio of only one dendritic cell per 200 periodate-treated lymphocytes. This evidence thus indicates strongly that in rats, dendritic cells—not macrophages—function as accessory cells. Further, the number of dendritic cells in a preparation governed the magnitude of the mitogenic response and was limiting in the case of unfractionated lymph node cells. In addition, when oxidized with periodate or with neuraminidase plus galactose oxidase, the dendritic cell served as a very potent indirect stimulator of untreated responder lymphocytes. Both functions of the dendritic cell appeared to lack species specificity, since mouse dendritic cells were very active when tested with rat responder lymphocytes. A soluble factor (accessory cell-replacing factor), produced by cultures of lymph node or spleen cells subjected to oxidative mitogenesis, enabled otherwise unresponsive mitogen-treated lymphocytes to respond. Dendritic cells were required for the production of this factor but may not be solely responsible for its production.**

It is widely recognized that the activation of T lymphocytes by mitogens requires the participation of accessory cells expressing Ia-antigens (1, 2). The identity of the accessory cell has not been rigorously established, although several authors have proposed that macrophages or monocytes can serve as accessory cells (3–7). Steinman and Cohn (8) have reported that preparations of adherent mouse spleen cells contain a new class of leukocyte, termed the "dendritic cell" because of its morphological characteristics. Not only do dendritic cells differ from macrophages morphologically, but also they lack Fc receptors, and they are not phagocytic. Cells having a dendritic appearance have been observed also in rabbit and rat (9, 10) lymphoid tissues.

In our previous work demonstrating an absolute requirement for accessory cells in oxidative mitogenesis of rat T lymphocytes, which will appear elsewhere, we have expressed reservations that macrophages serve as accessory cells in our system. The fact that mouse dendritic cells bear Ia-antigens and are extremely potent stimulators in a mixed leukocyte reaction (11) suggested that they might also function as accessory cells. Thus, we purified rat dendritic cells and found that they account for most, if not all, of the accessory cell activity of the starting lymphoid cell population. In addition, we showed that surface-oxidized dendritic cells are potent indirect stimulators of syngeneic responder lymphocytes. Finally, we demonstrated that dendritic cells play an obligatory role in the production of a soluble factor that is able to replace accessory cells in the mitogenic activation of T lymphocytes.

MATERIALS AND METHODS

Preparation of Lymph Node Cells (LNC). LNC were prepared from cervical and mesenteric lymph nodes of 150- to 225-g inbred male Lewis rats (Microbiological Associates, Bethesda, MD) as described (12). Cell concentrations were determined with a Coulter Counter (model ZBI, Coulter Electronics, Hialeah, FL).

Mitogen Treatment. Lymphocytes were treated with sodium periodate or with neuraminidase plus galactose oxidase (NGO) as described (12).

Fractionation of Cells in Bovine Serum Albumin Gradients. Washed cells (up to 1×10^8 cells per ml) were suspended in a dense ($\rho = 1.085$ g/cm^3) bovine serum albumin solution (Armour fraction V, Reheis Chemicals, Phoenix, AZ) prepared according to Steinman and Cohn (13). Suspensions were overlayered with 1 ml of light ($\rho = 1.048$ g/cm^3) albumin and centrifuged at 10,000 $\times$ g for 30 min. All procedures were carried out at 4°C. The resulting cell pellet and the cells at the albumin interface were collected with Pasteur pipettes. Cells were then washed twice in Hanks' balanced salt solution and counted. Total recovery of LNC ranged from 70–80%.

Removal of Adherent Cells. Cells were suspended (1×10^7 cells/ml) in RPMI 1640 medium (Associated Biomedics, Buffalo, NY) containing 10% (vol/vol) heat-inactivated horse serum (GIBCO). Cells [1×10^8 per 100-mm plastic tissue culture dish (no. 3003, Falcon)] were cultured for 2 hr at 37°C. Nonadherent cells were collected, and each dish was washed extensively with Hanks' balanced salt solution. Recovery of the total cells plated was 75–85%.

Removal of Fc Receptor-Positive Cells. Sheep erythrocytes were opsonized with a subagglutinating concentration of rabbit anti-sheep erythrocyte serum (a gift from R. Steinman). LNC and opsonized erythrocytes (EA) were resuspended in RPMI 1640 medium at a ratio of 1:50 and centrifuged to a pellet (375 $\times$ g, 10 min). Rosettes were allowed to form at 4°C for 30 min,

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Abbreviations: ACRF, accessory cell-replacing factor; IL, interleukin; LNC, lymph node cells; NGO, neuraminidase plus galactose oxidase; EA, opsonized erythrocytes.

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after which the cells were fractionated in a bovine serum albumin gradient, as described above, into rosetting (pellet) and nonrosetting (interface) cells (11).

Irradiation. Cells suspended in the salt solution were irradiated with 1000 rad (1 rad = 0.1 J/kg = 0.01 Gy) in a Gammacell 40 Small Animal Irradiator (Atomic Energy of Canada, Ottawa, Canada) at a dose rate of 114 rad/min.

Purification of Rat Dendritic Cells. The purification of rat dendritic cells was modified from the procedure of Steinman and Witmer (11). Direct adaptation was not feasible because the rat dendritic cell is a nonadherent cell, unlike the mouse spleen dendritic cell, which is adherent when first collected. Our procedure involved collecting the low-density population of rat LNC by fractionation in albumin. The cells were then γ-irradiated and cultured for 2 hr to remove most adherent cells. The nonadherent cells were returned to culture for 2–5 days, during which time the radio-sensitive cells died. The recovered cells were subjected to EA-rosetting to further reduce contamination by macrophages and other Fc receptor-bearing cells. During sedimentation of the erythrocytes, the nonviable cells pelleted with the rosetted cells. The final preparation, which floated to the albumin gradient interface, contained 70% to >90% dendritic cells with a yield of 0.01–0.1% of the starting LNC. The highest purity, with some sacrifice in yield, was obtained with preparations that had been cultured for 5 days.

Cell Culture and Assessment of Cellular Proliferation. Cells were routinely cultured in RPMI 1640 medium containing 10% heat-inactivated horse serum in a humidified atmosphere of air containing 7% CO_2. Proliferation of cultured lymphocytes was measured by the incorporation of [^{3}H]thymidine (Schwarz/Mann) between 44 and 48 hr after the start of the culture (14). The [^{3}H]thymidine incorporation data presented in this paper are the mean and standard deviation of triplicate values from representative experiments.

Assay of Accessory Cell Activity and Accessory Cell-Replacing Factor (ACRF). The assay for accessory cells and for ACRF was based on their ability to restore the mitogen-induced stimulation of accessory cell-depleted LNC. The LNC were fractionated in albumin as described above, and the sedimented cells (containing responder lymphocytes) were treated with periodate or with NGO. These test cells were cultured (5×10^5 cells per well) in a total volume of 0.2 ml, including either previously irradiated accessory cells or a sample of medium from ACRF-producing cultures. The medium had been collected 18–24 hr after the start of the culture period and freed of cells and debris by centrifugation and filtration through a sterile 0.22-μm membrane. As a rule, four to eight different amounts of accessory cells or of ACRF-containing medium were added. Stimulation was assessed on day 2 by measurement of [^{3}H]thymidine incorporation as described above. In the absence of added accessory cells (or ACRF), the control response was 50–400 cpm per culture. This is in comparison to a response of approximately 15,000 cpm for the same number of unfractionated, mitogen-treated LNC. As will be shown, accessory activity measured in this manner is directly proportional over a considerable range to the number of cells added or to ACRF concentration, providing the basis for a simple quantitative assay. One unit of accessory activity is defined as the amount producing a response 2000 cpm above the control response.

Indirect stimulation, which is defined as the stimulation of untreated lymphocytes by periodate- or NGO-treated cells [themselves blocked from proliferation by irradiation or mitomycin C (14)], was assayed by a similar procedure using untreated, accessory cell-depleted lymphocytes as responders, and periodate- or NGO-treated accessory cells as stimulators.

Electron Microscopy. Electron microscopy of the cultured cells fixed in glutaraldehyde and postfixed in osmium tetroxide was performed as described (15). Silver Epon sections were cut, doubly stained with uranyl acetate and lead citrate, and examined in a Philips EM-300 electron microscope operated at 80 kV.

RESULTS

Purification of Accessory Cells. After centrifugation of rat LNC in dense bovine serum albumin (ρ = 1.085), most cells sedimented, but a small portion (2–8%) floated and was recovered at the interface between the dense albumin and a less dense overlayer. As seen in Table 1, the sedimented cells did not proliferate after periodate treatment. Addition of irradiated, non-mitogen-treated cells from the floating population restored the proliferative response in a dose-dependent manner. From the slope of the line, an accessory activity of 0.24 milliunits per cell may be estimated for this population as compared with an activity of about 0.015 milliunits per cell for the starting LNC.

Among the cells readily identifiable in the floating population were small lymphocytes, lymphoblasts, plasma cells, and macrophages. Preliminary experiments indicated that the low-density population of LNC could be further enriched in accessory cell activity by removal of adherent cells, Fc receptor-bearing cells, radiation-sensitive cells, and such cells as became denser in culture. Conversely, the cells removed by these procedures had greatly reduced or no accessory cell activity. Therefore, these techniques were combined in a procedure for preparing highly purified accessory cells as outlined in *Materials and Methods*. A detailed account of the purification scheme and activity of the various cell preparations will appear elsewhere.

The highly purified accessory cell preparations generally contained 0.02–0.08% of the starting LNC and were extremely active in restoring the responses of periodate-treated lymphocytes (Table 2). Very few accessory cells were required to produce significant incorporation of [^{3}H]thymidine by 5×10^5 responders. The stimulation increased linearly with the number of added accessory cells and reached a maximum after addition of only 2900 cells (1 per 170 cells). The purified preparation shown in Table 2 had an accessory activity of about 16 milliunits per cell, indicating a 70-fold purification over the population of floating cells, and about 1000-fold greater than the starting LNC. Between 50% and 70% of the accessory cell activity of the starting material was recovered in the purified preparation.

Morphology of Purified Accessory Cells. Some 70–90% of the cells present in purified accessory cell preparations had the

Table 1. Accessory cell activity of the low-density population of rat LNC

Accessory cells*		
Number added	% of responder lymphocytes	[^{3}H]Thymidine incorp., cpm per culture
0	0	480 ± 30
3,120	0.6	2,720 ± 190
6,250	1.2	3,460 ± 550
12,500	2.5	6,840 ± 210
25,000	5	12,000 ± 810
50,000	10	23,900 ± 880
100,000	20	41,200 ± 720

* LNC were fractionated in a bovine serum albumin gradient. The low density "accessory cells" constituted 2.9% of the starting LNC.

Table 2. Accessory cell activity of highly purified rat accessory cells

Accessory cells*		[^{3}H]Thymidine incorp., cpm per culture
Number added	% of responder lymphocytes	
0	0	220 ± 10
12	0.0024	400 ± 30
36	0.0072	700 ± 130
108	0.022	1,750 ± 300
324	0.065	12,000 ± 2,800
972	0.194	30,800 ± 3,300
2920	0.583	55,300 ± 450
8750	1.75	54,000 ± 1,600

* Dendritic cells were purified as described in *Materials and Methods*. The final preparation contained 92% morphologically identifiable dendritic cells. The yield was 0.011% of the starting LNC.

features seen in the phase-contrast micrograph (Fig. 1, *Inset*). These cells are nonadherent and very motile, and they possess a highly irregular surface consisting of numerous projections that continually change shape. In an electron micrograph (Fig. 1) these projections can clearly be seen. An irregularly-shaped nucleus with condensed chromatin lining the nuclear membrane is also a characteristic feature. Rough endoplasmic reticulum is scarce; most ribosomes exist in clusters as free polysomes. Mitochondria, small membranous vesicles, and Golgi elements are evident, whereas lysosomes and secretory granules are less numerous. Generally, the cytoplasm in the dendritic

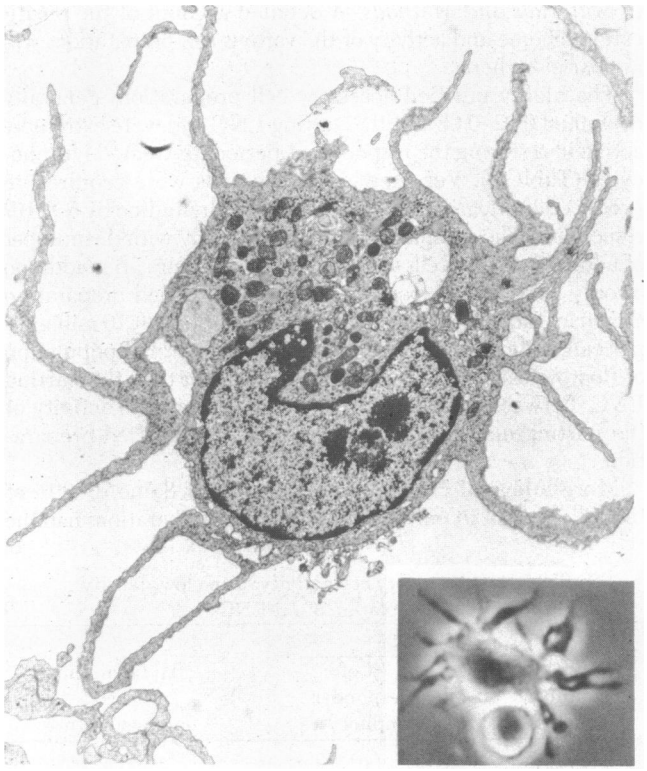

FIG. 1. Electron micrograph of a lymph node cell with typical dendritic morphological characteristics. Cells were purified and prepared for electron microscopy after 3 days in culture. The preparation contained 82% morphologically identifiable dendritic cells, and the overall yield was 0.05%. (×5000.) (*Inset*) Phase contrast photomicrograph of a dendritic cell and adjacent lymphocyte showing typical morphological characteristics in culture. Cells were purified without EA-rosetting after 5 days in culture, with an overall yield of 0.1%. (×800.)

Table 3. Accessory cell activity of mouse dendritic cells cultured with responder rat lymphocytes

Mouse dendritic cells*		[^{3}H]Thymidine incorp., cpm per culture	
Number added	% of responder lymphocytes	Untreated responder lymphocytes	Periodate-treated responder lymphocytes
0	0	50 ± 30	70 ± 20
2,500	0.5	100 ± 30	1,290 ± 30
5,000	1	120 ± 30	3,480 ± 530
10,000	2	200 ± 10	9,140 ± 950
20,000	4	490 ± 50	19,300 ± 280

* Purified mouse dendritic cells were added to 5×10^5 responder rat lymphocytes.

projections and in the zone adjacent to the cell membrane is devoid of most organelles. These features are characteristic of the mouse spleen dendritic cell (8). A cell having similar morphological characteristics has also been described in rat spleen (9) and has been termed the "interdigitating cell" (10).

Accessory Activity of Dendritic Cells in a Xenogeneic Mixture. A purified preparation of mouse (NCS, Rockefeller University strain) dendritic cells (a gift of M. Nussenzweig and R. Steinman) was tested for its ability to act as accessory cells with periodate-treated rat lymphocytes. The results in Table 3 show that excellent responses were obtained and that the mouse dendritic cells acted in a dose-dependent manner. In comparison, the same number of mouse dendritic cells, tested for their ability to stimulate untreated rat lymphocytes (a xenogeneic mixed leukocyte reaction), produced only low responses by the end of 2 days in culture.

Indirect Stimulation by Mitogen-Treated Dendritic Cells. Steinman and Witmer (11) have reported that in mice, dendritic cells provide the primary stimulus during a mixed leukocyte reaction. We found that rat dendritic cells are also very effective allogeneic stimulators (unpublished results). In view of our previous studies on indirect stimulation (12, 14, 16) and the parallels between it and the mixed leukocyte reaction, we wanted to determine if a mitogen-treated dendritic cell could also serve as the indirect stimulator cell in a syngeneic system—a role that has been attributed to macrophages (4). Highly purified rat dendritic cells were treated with NGO and added in small numbers to untreated, syngeneic responder lymphocytes. As seen in Table 4, extensive proliferation was elicited by as few as 1250 dendritic cells. After NGO treatment, mouse

Table 4. Indirect stimulation of untreated, accessory cell-depleted rat lymphocytes by NGO-treated rat or mouse dendritic cells

NGO-treated dendritic cells		[^{3}H]Thymidine incorp., cpm per culture	
Number added	% of responder lymphocytes	Rat cells*	Mouse cells†
0	0	80 ± 10	50 ± 30
312	0.06	230 ± 120	—
625	0.12	900 ± 280	—
1,250	0.25	1,560 ± 590	2,260 ± 320
2,500	0.50	3,050 ± 860	3,910 ± 180
5,000	1	7,230 ± 790	7,400 ± 530
10,000	2	13,200 ± 780	14,400 ± 90
20,000	4	—	24,400 ± 80

* A highly purified preparation of rat accessory cells was collected after 2 days in culture. The yield was 0.09% of the starting LNC.

† Gift from M. Nussenzweig and R. Steinman.

dendritic cells (the same preparation used in the experiments presented in Table 3) can also serve as indirect stimulators of rat lymphocytes (Table 4).

The Role of Dendritic Cells in the Production of ACRF. Our work (to appear elsewhere) indicated that cultures of mitogen-treated lymphoid cells contain a soluble factor, termed ACRF, that can can substitute for the required accessory cells. Having established that dendritic cells are the accessory cells in mitogenic stimulation, we investigated the role of dendritic cells in ACRF production.

Table 5 presents data concerning the amount of ACRF produced by cultures of various cells during the first 24 hr, which we previously found to be the period of maximal ACRF production by mitogen-treated LNC. No detectable ACRF (the limit of detection is 1–2 units/ml) was produced by untreated cells or, in most cases, by mitogen-treated responders. In contrast, the low-density population of LNC, which contains the dendritic cells, produced twice as much ACRF per cell when periodate-treated as unfractionated LNC did. However, this enhanced production did not correspond to the 10- to 20-fold enrichment in accessory cell activity obtained by the fractionation procedure. When periodate-treated responders, which make little or no ACRF by themselves, were mixed in culture with accessory cells (either untreated or periodate-treated), the total amount of factor produced by the mixture was much greater than the amount produced by the treated accessory cells alone.

To determine if the cell in the low-density population of LNC that produces ACRF is the dendritic cell, a preparation of highly purified rat dendritic cells was treated with NGO and cultured overnight. The medium was collected and assayed for ACRF (Table 5). These cells, which were predominantly dendritic in morphological appearance, produced more than 50 times as much ACRF per cell as did the low-density population of LNC. This correlates with the enrichment in dendritic cells generally obtained by the purification procedure.

Comparing the results of Table 5 with those of Tables 1 and 2, we find that we recovered from the cultures of either crude accessory cells or purified dendritic cells less than 5% of the accessory activity displayed by the cells themselves. Addition of responders increases the yield by a factor of 5. Because ACRF is stable, this suggests that interaction between the accessory cells and the responder cells causes the accessory cells to make more factor or induces a cell present in the responder population to produce ACRF.

Table 5. ACRF production by various cultured cells

Cells in the ACRF producing culture*		ACRF production†	
Type	Cells, no. $\times 10^{-6}$/ml	Units per 10^6 cells	Units/ml
Unfractionated‡	10	ND§	ND
IO_4-Unfractionated	10	2.4	24.2
Responder¶	10	ND	ND
IO_4-Responder	10	0.7	7.0
Accessory‖	2	ND	ND
IO_4-Accessory	2	4.8	9.5
IO_4-Responder with accessory	10 + 2	3.7	44.3
IO_4-Responder with IO_4-accessory	10 + 2	3.9	46.6
NGO-Dendritic**	0.3	253.0	75.9

* Cells were cultured overnight and the cell-free medium was collected for later ACRF assay. The IO_4 and NGO notations represent periodate- or NGO-treated cells.
† Assayed on periodate-treated, responder rat lymphocytes.
‡ Rat LNC.
§ ND = not detectable.
¶ The high-density population of rat LNC. In three other experiments, similar preparations of periodate-treated, responder rat lymphocytes produced *no* detectable ACRF.
‖ The low-density population of rat LNC.
** A highly purified accessory cell preparation, collected after 5 days in culture. The yield was 0.1% of the starting LNC.

DISCUSSION

The results described in this paper leave little doubt as to the identity of the cell responsible for the accessory activity in the mitogenic stimulation of rat T lymphocytes by either periodate or NGO. Our best purified preparations contained up to 92% typical dendritic cells and had more than 1000 times the specific accessory activity of the starting mixture. Most of the few nondendritic cells in the preparation were readily identified as transformed lymphocytes; macrophages were seldom detected. In all instances, removal of macrophages by a variety of procedures enhanced accessory activity. Purified macrophages from either lymph nodes or peritoneal exudates, on the other hand, were essentially inactive (unpublished data).

Therefore, it may be confidently stated that rat macrophages are not the bearers of accessory activity. Most probably, the dendritic cell is solely responsible for this activity. Our results apply only to the rat, whereas other investigators have worked with human, mouse, and guinea pig cells. However, we also found that purified mouse spleen dendritic cells were able to function as accessory cells with rat responder lymphocytes. Investigators (3–7) who claimed a requirement for macrophages as accessory cells used very high ratios of macrophages to lymphocytes (1:1 to 1:5) in order to obtain maximal responses. In view of their potency, a small number of dendritic cells contaminating these macrophage preparations could easily account for such findings.

A similar explanation may account for the reported ability of macrophages to act as indirect stimulators in oxidative mitogenesis (4). Indirect stimulation by oxidized dendritic cells is of interest because the dendritic cell is a potent stimulator in the allogeneic mixed leukocyte reaction in both the mouse (11) and the rat (unpublished observation). It is possible that indirect stimulation could serve as a polyclonal model for the cell–cell interactions that occur in a mixed leukocyte reaction.

When enough accessory cells were added, the response of T lymphocytes could be increased well above the response of unfractionated LNC (about 15,000 cpm with 5×10^5 cells) up to a maximum level some 4- to 5-fold higher. Because [^{3}H]-thymidine incorporation under our labeling conditions correlates with the number of responding lymphocytes as determined by autoradiography (14), the plateau most likely represents a saturation by dendritic cells of all lymphocytes capable of responding. Thus, our results suggest that the magnitude of mitogen-induced responses depends on the number of dendritic cells in the preparation.

Growth factors that enhance T lymphocyte responses have recently (17) been categorized into two general classes termed "interleukins" (IL). IL 1, most frequently referred to as lymphocyte-activating factor (18), has generally been thought to be produced by macrophages and is required for the production of IL 2 (thymocyte-stimulating factor) (19) by helper T cells (20). We use the phenomenological term "ACRF" to describe the soluble factor studied in these experiments, because our assay would not distinguish between IL 1 and IL 2 activities (20). It is clear from the results presented in Table 5 that ACRF is produced only in cultures that contain dendritic cells, and it is likely that the dendritic cell does make some ACRF. Less clear is whether the dendritic cell is the sole ACRF-producing cell.

Possibly, the ACRF activity produced by purified dendritic cells is of the IL 1 type, but when the dendritic cells are cocultured with helper T cells present in the responder lymphocyte population, a mixture of both IL 1 and IL 2 is produced. Although consistent with other data, this interpretation needs to be substantiated by additional experiments.

We have also observed that stimulation induced by another T cell mitogen, concanavalin A, is also dependent upon the presence of dendritic cells (data not presented). However, it appears that fewer dendritic cells are required to support concanavalin A stimulation, because two sequential bovine serum albumin fractionation steps are occasionally needed to completely eliminate the response of LNC to this mitogen. ACRF also is produced in cultures of concanavalin A-stimulated LNC or spleen cells. Thus, our findings may be of general significance for T cell stimulation.

In summary, our results demonstrate that the dendritic cell, not the macrophage, is the obligatory accessory cell in oxidative mitogenesis of rat T lymphocytes. Because of the likelihood that macrophage preparations may contain small proportions of potent dendritic cells, immunological functions previously attributed to macrophages must be reinvestigated.

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