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Clustering with dendritic cells precedes and is essential for T-cell proliferation in a mitogenesis model

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SUMMARY

Several antigen-specific immune responses are known to occur in discrete aggregates of dendritic cells (DC) and lymphocytes. We have used a polyclonal model, the mitogenesis of T cells that have been modified with sodium periodate, to evaluate the significance of cell–cell clustering. Firstly, we found that clustering precedes the onset of DNA synthesis by a day. Within 2 hr, virtually all of the added dendritic cells and most of the T cells that will respond have formed clusters. The T cells then progressively release and become responsive to interleukin-2 over 18 hr and DNA synthesis begins at 24 hr. Secondly, clustering with dendritic cells appears to be essential for mitogenesis. If dendritic cells are eliminated, the clusters disassemble and subsequent proliferation is reduced. Clustering and proliferation can be restored with dendritic cells that are syngeneic or allogeneic with the initial inoculum. DC are inactive if they are treated with ultraviolet light, formaldehyde or heat. Thirdly, the non-clustered cells do not synthesize DNA even when mixed with the clusters. However, non-clusters will respond when supplemented with additional DC. We conclude that clustering with DC precedes and seems essential for T-cell mitogenesis in the periodate model.

INTRODUCTION

The early events in the activation of resting T lymphocytes are typically studied using mitogens rather than specific antigens as stimuli, since relatively large numbers of cells enter into cell cycle. Mitogen responses, like those induced by antigen, require accessory cells. In oxidative mitogenesis, which is the proliferation of T cells that have been modified with sodium periodate, or with neuraminidase-galactose oxidase, the dendritic cell (DC) is the principal accessory cell (Klinkert *et al.*, 1978; Phillips *et al.*, 1980b; Van Voorhis *et al.*, 1983; Austyn *et al.*, 1983). Our studies in the murine system have indicated that mitogenesis proceeds in two stages. During the first 18 hr or so, DC induce T cells to release and become responsive to the growth factor, interleukin-2 (IL-2). These responsive T cells can then proliferate in response to IL-2, without the continued presence of DC (Austyn *et al.*, 1983). Events occurring in the first stage have now been analysed in more detail. DC and responding T cells interact rapidly with one another to form discrete cell aggregates within 2 hr of co-culture, which is at least 20 hr prior to the onset of DNA synthesis. We describe several variables in the aggregation phenomenon and present data that clustering with DC is essential for mitogenesis.

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Abbreviations: C, complement; c.p.m., counts per minute; DC, dendritic cells; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; IL- , interleukin; MHC, major histocompatibility complex.

MATERIALS AND METHODS

Mice

CD2 F_1 (H-2d), Swiss (H-2s), B6.H2k (H-2k) and C3H/He (H-2k) mice were from the Trudeau Institute, Saranac Lake, NY. Mice were of both sexes and 6–12 weeks of age.

Culture media

Medium used throughout was RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5–10% heat-inactivated fetal calf serum (KC Biologicals, Lenexa, KS), 2-mercaptoethanol (5×10^{-5}M; Sigma Chemical Co., St Louis, MO) and antibiotics (20 μg/ml gentamycin sulphate; Schering Corp., Kenilworth, NJ). Cells were washed with RPMI-1640 or, where indicated, with PBS which is phosphate-buffered saline without calcium and magnesium (Gibco).

Cells

Murine leucocytes were prepared and treated as described elsewhere (Austyn *et al.*, 1983).

Dendritic cells. Enriched populations were from low-density, spleen-adherent cells, from which most macrophages were depleted by re-adherence to tissue culture dishes. In some

experiments the DC were exposed to UV light (10 cm from a General Electric 15 watt G15T8 lamp), to 0·01% formaldehyde in PBS, or incubated at 44° for 20 min prior to use.

T lymphocytes. T cells (>96% pure) were from spleen cells, sometimes supplemented by those from mesenteric lymph nodes, that were passed over nylon-wool columns and treated with ammonium chloride to lyse erythrocytes. They were then treated with anti-Ia antibody and rabbit serum as a source of complement (C). For periodate modification, T cells were washed in PBS and treated with sodium *m*-periodate, as described elsewhere.

Oxidative mitogenesis

Proliferation assays. Unless stated, stimulator cells were irradiated (2000 rads from ^{137}Cs; Gammacell 1000, Atomic Energy of Canada Ltd, Ottawa, Canada) and added in graded numbers to $2{\cdot}5\text{–}5 \times 10^5$ periodate-treated T cells in a total volume of 200 μl in flat-bottomed microtitre plates (3596; Costar, Cambridge, MA). Except for the experiment in Fig. 1, 0·5–1 μCi/well (=18·5–37 KBq/well) of tritiated thymidine (6·7 Ci/mmol=247·9 GBq/mmol; Schwartz/Mann, Orangeburg, NJ) was added at 24–28 hr after the start of culture (this total time of culture is exclusive of any interruptions for treatment of the cells, see below) and cells were harvested 14–18 hr later. Results are the means (c.p.m.) of triplicate cultures, and SD were routinely <15% of the mean for values >1000 c.p.m.

Cytotoxic elimination of DC. DC were mixed with periodate-modified T cells as indicated, and distributed into 16 mm wells at $0{\cdot}8\text{–}1 \times 10^7$/well in 1 ml medium. At various times (3–18 hr), the cultures were harvested and treated with 33D1 anti-DC (Nussenzweig *et al.*, 1982), B21-2 anti-$Ia^{b,d,s}$ (Steinman *et al.*, 1980) or 10-2.16 anti-Ia^k (Oi *et al.*, 1978) monoclonal antibodies together with rabbit C. Isolated clusters (see below) were similarly treated. The cells were distributed into microculture wells at $2\text{–}5 \times 10^5$/well and 200 μl total volume. The cultures were maintained in fresh medium, 50% supernatant from 18 hr oxidative mitogenesis cultures, or DC as shown.

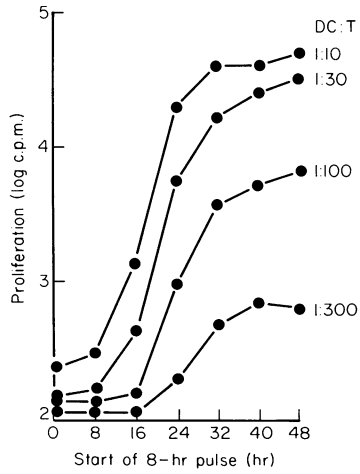

Figure 1. The kinetics of DNA synthesis during oxidative mitogenesis. Periodate-modified T cells were cultured with graded doses of DC in replicate cultures and proliferation was measured at 8-hr intervals: tritiated thymidine, as described in the Materials and Methods, was added at the indicated times (i.e. 0 hr, 8 hr, 16 hr, etc.), and the cells were harvested 8 hr later (i.e. 8 hr, 16 hr, 24 hr, etc., respectively).

Fractionation of clusters and non-clustered cells. DC were mixed with periodate-modified T cells and distributed into either 16 mm wells or 15 ml conical tubes: clusters formed more quckly in the latter. At the indicated times the cells were gently resuspended and harvested for fractionation in Percoll gradients, as described elsewhere (Inaba & Steinman, 1984). The clustered and non-clustered cells, with or without treatment with antibody and C, were recultured in microtitre wells at 2×10^5 per well and pulsed as above.

RESULTS

Kinetics of T-cell proliferation and activation during oxidative mitogenesis

Graded doses of DC were added to a constant number of periodate-modified T cells, and tritiated thymidine uptake was measured at 8-hr intervals. The level of DNA synthesis was dependent upon the dose of DC and began at about 24 hr (Fig. 1). Proliferative responses also occurred if the T cells were not periodate modified, but this syngeneic mixed leucocyte reaction was typically less than one-tenth the oxidative mitogenesis response and peaked on the fifth day of culture.

We have shown previously that T-cell activation during oxidative mitogenesis involves both the release of and responsiveness to IL-2 (Austyn *et al.*, 1983). To monitor the acquisition of IL-2 responsiveness, graded doses of DC were again mixed with a constant number of T cells, but cultured for 3, 8 or 18 hr. At these times, replicate cultures were harvested and treated with or without a cytotoxic anti-Ia antibody and C to eliminate the DC. This treatment markedly inhibited the subsequent

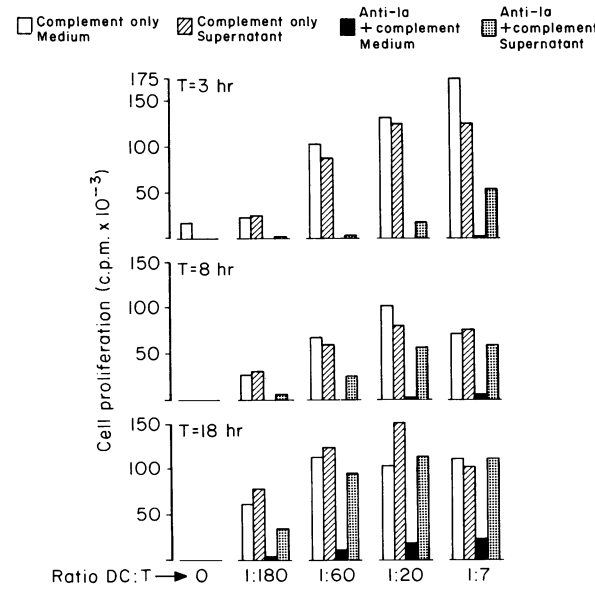

Figure 2. The kinetics with which DC induce T cells to become responsive to IL-2 in oxidative mitogenesis culture supernatant. Periodate-modified T cells were cultured with graded doses of DC in 16 mm wells. At 3 hr, 8 hr or 18 hr, the cultures were harvested and treated with anti-Ia antibody and complement to kill the DC, or with complement alone. The treated cells were than maintained in fresh medium or an IL-2-rich supernatant from an 18-hr culture before measuring DNA synthesis.

Table 1. The T cells that proliferate during oxidative mitogenesis are found in clusters

Exp.*	Time(hr)†	Cells	Proliferation‡
I	2	Clusters	137,810
		Non-clusters	3895
		T only	925
		Unseparated	58,641
		Mix 50:50§	94,715
		40:60	69,056
		20:80	37,209
IIA	3	Clusters	56,042
		Non-clusters	1817
		T only	152
IIB	6	Clusters	163,045
		Non-clusters	195
		T only	577
IIIA	8	Clusters	73,638
		Non-clusters	409
		T only	225
		Unseparated	43,971
		Mix 33:66§	38,682
IIIB	8	Clusters	98,709
		Non-clusters	2203
		T only	225
		Unseparated	65,609
		Mix 33:66§	66,213
IV	14	Clusters	76,255
		Non-clusters	1747
		T only	322
		Unseparated	46,557
		Mix 33:66§	30,942

* Four representative experiments in which DC and periodate-treated T cells were initially (Time 0) co-cultured at a ratio of 1:50, except Exp. IIIB where this was 1:20.

† Cells were harvested at the times shown, and either left unseparated or fractionated by velocity sedimentation into cluster and non-cluster fractions.

Proliferation (c.p.m.) of 2×10^5 of the indicated cells after a total time in culture (i.e. excluding the time for treatment) of 24–28 hr.

§ Clusters and non-clusters, respectively, were mixed in the indicated ratio, as a titration (Exp. I) or according to the yield from the gradient (Exps III and IV). The yield of clusters was about 40% of the total recovered cells. In Exps I, IIIB and IV, 40% of the proliferative response of the clusters would account for the proliferation of the unseparated cells or the mixture of clusters and non-clusters.

proliferative response in fresh medium (Fig. 2, compare first and third bars in each group). However, replicate cultures could proliferate in the presence of conditioned medium (Fig. 2, compare first and fourth bars in each group). The latter was obtained from DC–T-cell co-cultures that were previously shown to be a rich source of IL-2. Responsiveness to conditioned medium increased progressively with increasing time of co-culture and dose of DC (Fig. 2). In subsequent experiments, 10 U/ml recombinant human IL-2 gave similar effects (not shown). Full restoration of T-cell responses, to the level observed in the presence of intact DC, was obtained if T cells

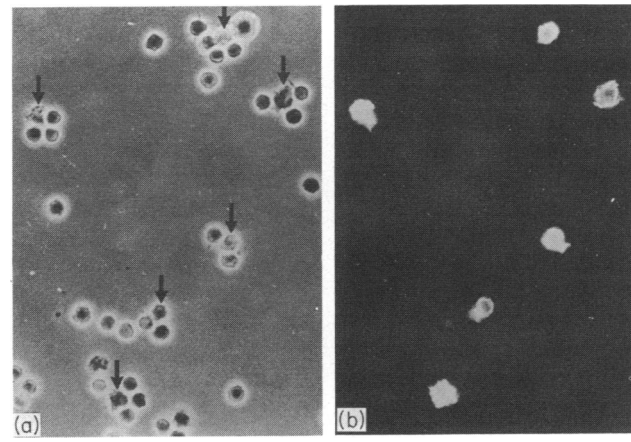

Figure 3. Identification of Ia-positive DC in cell clusters. Clustered cells were isolated at 2 hr from cultures of B6.H-2k DC (which are all IA^k positive) and periodate-modified allogeneic CD2 F_1 T cells. The clusters were attached in serum-free medium to coverslips that had previously been coated with 50 g/ml poly-L-lysine. (a) Phase-contrast view; (b) immunofluorescence of the same field after staining with monoclonal anti-Ia^k antibody, 10-2.16, and FITC goat anti-mouse Ig (Cappel) both at 4°. The DC (arrows in phase contrast) stain brightly and can be enumerated with ease. In two experiments the yield of clusters to non-clusters was between 1:2 and 1:3; the proportion of Ia-positive cells in unseparated, clustered and non-clustered cells was, respectively, 4%, 13% and < 1%. Monoclonal antibodies to Mϕ and B cells did not stain the clusters (not shown).

were cultured 8 hr with 15% DC or 18 hr with 5% DC. T cells that were cultured in the absence of DC did not become IL-2 responsive (Austyn *et al.*, 1983; and below). The IL-2-rich conditioned medium did not enhance the proliferation of DC–T cell mixtures that had been treated with C alone (Fig. 2, compare first and second bars in each group) so that an optimal amount of endogenous IL-2 was normally released into the medium. We conclude that responsiveness to IL-2 is acquired steadily over an 18 hr period, and then DNA synthesis begins.

The proliferating T cells are present in discrete clusters

Previous studies of the MLR and T-dependent antibody responses had shown that DC and responding T cells formed clusters after 1–2 days of co-culture (Inaba *et al.*, 1983, 1984; Inaba & Steinman, 1984). Cell clusters also developed in the oxidative mitogenesis response. The aggregation was rapid (1–2 hr), thereby providing an opportunity to study the relationship between clustering and subsequent entry into the cell cycle.

Clusters were separated from non-clustered cells by velocity sedimentation after 2–14 hr of DC–T cell co-culture, and subsequent proliferation was assessed. The cells isolated in clusters represented about 40% of the total when the initial ratio of DC:T was 1:30–1:50. The proliferative activity was confined to the cluster fraction at all time points, and even as early as 2 hr (Table 1). In 12 consecutive experiments, proliferation of the non-clustered cells was $5{\cdot}2 \pm 6{\cdot}6\%$ of the corresponding clustered component. When clustered and non-clustered cells were mixed, the response was similar to that of the unfractionated cultures (Table 1; experiments I, III and IV). The proliferative response in such mixtures could be attributed almost entirely to the cluster component (Table 1, legend). At the doses used,

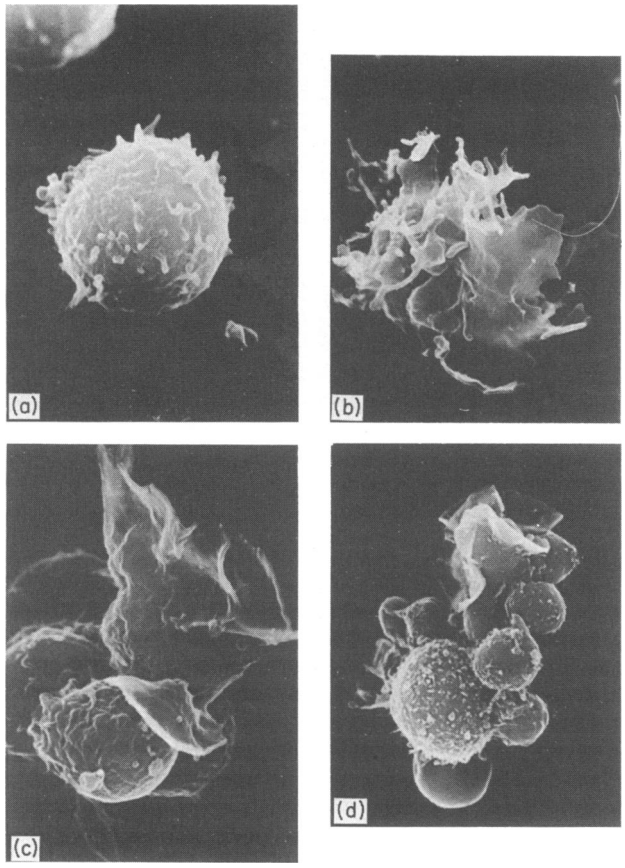

Figure 4: Scanning electron micrographs of DC, T cells and DC–T cell clusters. Cells were fixed in 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer, pH 7·4, at 37° and processed as described (Pugh *et al.*, 1983). They were examined on a JEOL 100CX electron microscope. (a) Periodate-treated T cells (× 18,900); (b) DC (× 18,900); (c, d) 4-hr DC–T-cell clusters (× 15,125 and × 5670, respectively (a single, spherical lymphoblast is visible in the latter).

clusters accounted for 30–40% of the cultured cells. This proportion of DNA synthesis in the enriched aggregates was equivalent to that of the unfractionated cultures. Taken together, these findings indicate that the bulk of the proliferative response emanates from T cells that form early clusters.

DC are also localized to clusters

We next tested if the partitioning of immune respossiveness between cluster and non-cluster fractions could be attributed to the physical partitioning of accessory cells. DC were enumerated in the fractions by immunofluorescence. For these experiments we used allogeneic mixtures of H-2k DC and H-2d T cells that gave very similar responses to syngeneic combinations (see below). The DC could be brightly labelled with a specific anti-Ia^k antibody and were found only in the clusters (Fig. 3). The number of DC in the cluster fraction was comparable to that added initially to the culture (Fig. 3 legend). The DC-specific antibody 33D1 was also tested in these immunofluorescence studies but the staining was too weak to enumerate DC.

Cluster and non-cluster fractions were also evaluated by scanning electron microscopy, since the surface topography of DC, which includes numerous veils of cytoplasm, was readily distinguished from that of the lymphocytes (Fig. 4, a, b). The DC were found primarily in the cluster fraction, closely associated with the spherical lymphocytes (Fig. 4, c, d). We conclude that DC are physically associated with responding T cells during oxidative mitogenesis.

Table 2. DC are required for proliferation of T cells in clusters

Cells and treatment*	Proliferation (c.p.m.): Syn. H-2s DC:H-2s T 3 hr	6 hr	9 hr	Allo.H-2k DC:H-2s T 3 hr	4 hr
Clusters					
No Ab/C	40,653	62,632	58,253	25,708	68,230
Anti-DC/C	2188	ND	9213	12,745	42,698
Anti-Ia/C	230	588†	1435	505	3240
Non-clusters	2845	ND	5153	5563	1075
T cells	385	548	598	1355	1935

* Clusters were isolated at the indicated times after mixing DC and periodate-treated T cells. They were treated with C, with or without anti-DC or anti-Ia antibody B21-2 (first three experiments) or 10-2.16 (last two experiments) specific for the stimulator haplotype. They, or non-clustered cells or T cells, were independently returned to culture and thymidine was added after a total period of culture of 24–28 hr and proliferation measured as described in the Materials and Methods. There was no reproducible difference in proliferation between cells treated with complement alone (first line), and those treated without or with heat-inactivated complement (not shown).

† In this experiment, supplementing with IL-2 or with DC, respectively, boosted the response of anti-Ia-treated cells to 26,532 and 50,413 c.p.m., and of T cells alone to 2323 and 24,294 c.p.m.

ND, not done.

Table 3. Non-clustered cells proliferate when supplemented with DC

Exp. 1		Exp. 2		Exp. 3	
%DC	C.p.m.	%DC	C.p.m.	%DC	C.p.m.
0	5588	0	1213	0	3895
0·5	21,905	0·3	1808	0·03	13,112
1	40,870	1	5620	0·1	21,547
2	50,298	3	20,130	0·3	44,238
4	90,805	10	59,123	1·0	106,651
				3·0	189,078

Non-clustered cells were isolated at 2 hr (Exp. 3) or 3 hr (Exps 1 and 2). Note that Exp. 3 utilized the same cells as in Table 1, Exp. 1.

DC are required for proliferation of clustered T cells

In order to evaluate the contribution of DC to proliferation of the clustered T cells, the clusters were treated with an anti-Ia

Table 4. The function of DC in oxidative mitogenesis is not MHC restricted

	Proliferation (c.p.m.) when reconstituted with						
		H-2k DC			H-2s DC		
Treatment	Medium	1×10^3	3×10^3	1×10^4	1×10^3	3×10^3	1×10^4
Cluster no Ab/C	59,050	64,853	85,783	103,055	68,178	78,735	88,025
Cluster anti-DC/C	19,008	21,318	32,993	73,580	23,128	32,803	69,780
Cluster anti-Ia/C	468	4495	18,573	57,083	4535	17,435	54,515
Non-cluster	205	2523	17,603	62,153	2558	13,855	59,870
T cells	293	13,675	44,415	97,643	15,193	53,670	102,415
Unseparated no Ab/C	16,113	20,045	30,493	45,893	23,108	30,435	62,838
Unseparated anti-DC/C	6748	10,458	25,288	33,033	10,945	20,938	47,240
Unseparated anti-Ia/C	130	1148	8818	35,070	400	5183	33,260

Cultures of H-2k DC with H-2s periodate-modified T cells were mixed and cultured for 6 hr before treatment with antibody (Ab) and complement (C) as shown. They were returned to culture in fresh medium (without IL-2) with or without DC that were allogeneic (H-2k) or syngeneic (H-2s) to the T cell.

antibody and C to kill the DC. This lead to their disassembly and largely ablated subsequent proliferative activity (Table 2). This was true for both syngeneic and allogeneic cell mixtures, the latter being employed to restrict the effect of anti-Ia and C treatment to DC rather than the responding T cells. The specific anti-DC antibody 33D1 and C substantially, but not fully, disaggregated the clusters and the ensuing proliferative response was decreased in most experiments (Table 2). Presumably some of the clustered Ia-positive cells had become resistant to 33D1 killing, since the co-cultures were initially (Time 0) more sensitive to this treatment (Austyn *et al.*, 1983). Proliferation was restored completely when fresh DC were added to DC-depleted clusters (e.g. Table 2 legend, and Table 4 below).

Mixing experiments (see above) had suggested that non-clustered T cells did not proliferate when they were cultured with clusters (Table 1). Since this indicated in turn that non-clusters did not respond to soluble factors, we asked if proliferation of the non-clustered fraction could be restored with additional DC. Marked responses were observed (Table 3), suggesting that the initial number of DC was insufficient to cluster and activate all the responsive T cells.

Requirements for DC-mediated clustering and T-cell activation

We found that treatment of DC with aldehyde fixatives, heat or UV light, which are known to ablate the function of DC, totally prevented clustering and oxidative mitogenesis, and no clustering occurred at 4° (not shown).

To test if the DC–T cell interaction required recognition of polymorphic products of the major histocompatibility complex (MHC), we mixed H-2k DC with periodate-modified H-2s T cells, separated clusters at 6 hr, eliminated the DC with either anti-Ia^k or anti-DC antibodies and C, and tested the ability of H-2k and H-2s DC to restore proliferation. DC from both strains formed clusters with the T cells (not shown) and were equally effective in restoring mitogenesis (Table 4). Similar results were obtained with unfractionated cultures (mixtures of clusters and non-clusters) as well as non-clustered and fresh T cells (Table 4). Thus, T cells which have bound to DC of one MHC can also bind and respond to DC of another MHC, as expected for a true polyclonal response.

DISCUSSION

It is becoming clear that DC are required to activate resting T cells to become lymphoblasts. These sensitized T cells can then be stimulated by other cell types bearing the relevant MHC molecules to which they are restricted (Inaba & Steinman, 1984). In a number of antigen-specific immune responses, the bulk of the DC and responding lymphocytes are associated in cell aggregates or clusters that are typically five to 15 cells in diameter. These clusters can be separated from non-clustered cells by velocity sedimentation. When returned to culture, DC and T cells remain associated for two or three more days and generate immune responses that include T-cell proliferation, lymphokine release and helper T–B lymphocyte interactions (Inaba *et al.*, 1983, 1984; Inaba & Steinman, 1984).

The analysis of clustering in primary antigen-specific responses has been demanding. The frequency of antigen-specific T cells is so small that one has to add a relative excess of DC to ensure that contact with antigen-reactive cells occurs; the aggregates have only been isolated after 1–2 days of culture; and comparatively few clusters are obtained. Thus it has not been possible to study the initial cell–cell interaction and even to prove that it is necessary for subsequent T-cell development. The oxidative mitogenesis model has overcome many of these limitations, and has been used in this paper to demonstrate several new features of the clustering phenomenon.

The first is that DC–T-cell clustering can precede the onset of DNA synthesis by at least 18 hr. When clusters were isolated after just 2–3 hr of co-culture at 37°, the bulk of the DC (Figs 3 and 4) and responsive T cells (Tables 1 and 2) were aggregated. The acquisition of IL-2 responsiveness occurred progressively between 2 and 18 hr (Fig. 2) and DNA synthesis began at 24 hr (Fig. 1). The second finding is that clustering with DC seems essential for T-cell mitogenesis. If DC were removed at 2–18 hr (Tables 2 and 4, Fig. 2) proliferation ceased. The proliferative

response in unseparated cultures can be accounted for by that of the cluster component (Tables 1 and 4). Thirdly, however, the non-clustered cells could cluster and proliferate if additional DC were provided (Table 3), and the failure of non-clustered cells to proliferate was thus not due to a lack of mitogen. All of these observations stand in contrast to findings with clustered and non-clustered cells isolated at 2 days from antigen-specific responses: the former are specifically enriched and the latter correspondingly depleted, for antigen-reactive cells (Inaba *et al.*, 1984). The mixing data (Table 1) also indicate that mitogenesis of the non-clustered cells is not induced by factors released from the clusters.

Finally, proliferation was restored in DC-depleted cultures equally if syngeneic or allogeneic DC were added back (Table 4) and we conclude that oxidative mitogenesis is not restricted by polymorphic MHC determinants. Similar conclusions were reached by others: Greineder & Rosenthal (1975), for instance, showed that syngeneic and allogeneic accessory cells, presumed to be macrophages, were equally effective in the guinea-pig model, while xenogeneic cells were not. Others have reported inhibition of oxidative mitogenesis with anti-Ia antisera in the guinea-pig (Greineder, Shevach & Rosenthal, 1976) and in the mouse (Phillips *et al.*, 1980a). In preliminary experiments we found likewise, but anti-class I antisera also inhibited (not shown); conceivably this was due to inhibition of proliferation of class I-restricted T cells in this system (further data to support this idea are in a subsequent report: Austyn & Morris, 1988.

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