

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/27180>

Please be advised that this information was generated on 2017-12-05 and may be subject to change.

JIM 06455

A rapid isolation procedure for dendritic cells from mouse spleen by centrifugal elutriation

Miriam A. Ossevoort ^a, René E.M. Toes ^a, Marloes L.H. De Bruijn ^b, Cornelis J.M. Melief ^a,
Carl G. Figdor ^b and W. Martin Kast ^a

^a *Department of Immunohematology and Bloodbank, University Hospital, Leiden, Netherlands, and* ^b *Division of Immunology, The Netherlands Cancer Institute, Amsterdam, Netherlands*

(Received 17 December 1991, revised received 20 March 1992, accepted 19 May 1992)

The standard isolation procedure for antigen presenting dendritic cells (DC) takes 2 days and includes selective adherence to tissue culture plates which may lead to the activation of these cells. This report describes the isolation of DC by centrifugal elutriation (CE). Murine spleen cells were separated on the basis of size and density into 7 CE fractions. This method took 90 min. Cells from each CE fraction were characterized by fluorescence activated cell sorter (FACS) analysis and their antigen presenting cell (APC) activity was determined by a secondary Sendai virus specific T cell proliferation assay. CE fraction 5 contained most of the DC with a concentration of 6–10%, representing an approximately 15-fold enrichment compared to unseparated spleen cells (<1% DC). This CE fraction also exhibited the highest APC activity, which was almost completely abolished after depletion of DC by treatment with monoclonal antibody 33D1 (DC-marker) and complement. Further enrichment of CE fraction 5 by discontinuous density gradient centrifugation resulted in a cell population containing 35–55% 33D1-positive cells with similar characteristics as DC isolated by the standard procedure, such as the capacity to induce a primary viral peptide specific CTL response. Two-color FACS analysis showed an increase in MHC expression on 33D1-positive cells of CE fraction 5 after 18 h culture involving cell adhesion to a similar level as the MHC expression on DC isolated by the standard procedure. During this same period their morphology changed from a round to a dendritic appearance. In conclusion, our results indicate that CE is well suited for isolating DC more rapidly and without activation of these cells by adherence, a process which readily occurs in the standard isolation procedure.

Key words: Centrifugal elutriation; Dendritic cell; Antigen presenting cell; Mixed lymphocyte reaction; (Spleen); (Mouse)

Correspondence to: W.M. Kast, Department of Immunohematology and Bloodbank, University Hospital Leiden, Building 1, E3-Q, P.O. Box 9600, 2300 RC Leiden, Netherlands. Tel.: 31-71-263800; Fax: 31-71-216751.

Abbreviations: APC, antigen presenting cell; CE, centrifugal elutriation; DC, dendritic cell.

Introduction

Dendritic cells (DC) are irregularly shaped, extremely potent accessory cells involved in the induction of T cell responses (Steinman et al., 1983; Boog et al., 1985; Austyn, 1987; Kast et al., 1988; Knight et al., 1988; Macatonia et al., 1989;

Melief, 1989; Inaba, 1990; King, 1990). DC do not proliferate *in vitro*. For many years the isolation procedure has been a major limiting step in the investigation of the functional and phenotypic properties of DC. The standard isolation procedure of DC takes 2 days and includes an 18 h culture period involving cell adhesion to tissue culture plates (Steinman et al., 1979). Adherence may induce biological and biochemical changes in DC which may, in turn, lead to the activation of these cells as has been described for monocytes (Figdor et al., 1986; Kelley et al., 1987; Te Velde et al., 1990). Activation of cells by adherence can be avoided by centrifugal elutriation (CE). Cell populations are separated by CE into different fractions on the basis of size and density. CE is used widely for the separation of different cell populations (Pretlow and Pretlow, 1979). Thompson et al. (1983) have described a method for size separation of murine spleen cells using CE. CE has also been used to isolate hemopoietic stem cells (Nijhof et al., 1984; Ploemacher et al., 1987) and size-dependent B cells (Thompson et al., 1984; Jelachich et al., 1986) from murine spleen cells. Here, CE has been used for the first time to isolate murine DC. Murine spleen cells were separated into seven CE fractions within 90 min. By FACS analysis and analysis of antigen presenting cell (APC) activity most DC were shown to be present in one particular CE fraction. After further enrichment the functional and phenotypic properties of these CE isolated DC were determined.

Materials and methods

Animals

C57BL/6 (B6) mice were bred under specific pathogen-free conditions at the Netherlands Cancer Institute, Amsterdam, Netherlands.

Virus and immunization

Non-virulent Sendai virus, lot 40340089 was obtained from Flow Laboratories (McLean, VA, USA) and stored at -70°C . Mice were primed by one intraperitoneal injection of 10^2 hemagglutinating units (HAU) of non-virulent Sendai virus at the Central Laboratory of The Netherlands

TABLE I

MONOCLONAL ANTIBODIES USED IN THIS STUDY

Antibodies	Specificity	Reference
33D1	Lymphoid DC	Nussenzweig et al. (1982)
RA3.6B2	B220	Morse et al. (1982)
Mac-2	Macrophage	Ho et al. (1982)
F7D5	Thy-1.2	Lake et al. (1979)
50.Ad22/15	Thy-1	Ledbetter et al. (1979)
Anti-asialo G_{M1} ^a	NK cell	Kasia et al. (1980)
H141.31.10	H-2D ^b	Lemke et al. (1979)
17-227	I-A ^{k,b,d}	Sachs et al. (1981)

^a Antiserum.

Red Cross Blood Transfusion Service, Amsterdam, Netherlands (De Waal et al., 1983). Spleen cells were used 2–6 weeks after immunization.

FACS analysis

All procedures were performed at 4°C in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (Boehringer Mannheim, Germany) and 0.02% NaN_3 . Erythrocytes were removed by treatment with NH_4Cl .

Spleen cells were incubated for 30 min with saturating concentrations of monoclonal antibody or antiserum (Table I) in 96 well V bottomed plates (Greiner, Langenthal, Switzerland). Cells were washed twice by centrifugation ($270 \times g$, 3 min), incubated for 30 min with saturating concentrations of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse (GAM) $\text{F(ab}')_2$ FITC (Tago, Burlingame, CA, USA), mouse anti-rat κ (MARK) FITC (Sanbio, Uden, Netherlands) or rabbit anti-rat (RAR) FITC (Organon Teknika, Boxtel, Netherlands) antibodies, washed twice, resuspended and analyzed for fluorescence. Alternatively, cells were incubated for 30 min with FITC-labeled antibodies, washed twice, resuspended and analyzed for fluorescence. For two-color fluorescence, cells were incubated for 30 min with a mixture of FITC-labeled antibody and biotin-labeled 33D1 at concentrations predetermined to be saturating. Cells were washed twice by centrifugation, incubated for 20 min with $10 \mu\text{l}$ streptavidin phycoerythrin (SA-PE, Becton and Dickinson, Mountain View, CA, USA), again washed twice, resuspended in PBS-BSA-Az supplemented with 0.2% paraformaldehyde and ana-

lyzed for fluorescence. Cytofluorographic analysis was performed using a FACScan flow cytometer with consort 30 software (Becton and Dickinson, Immunocytometric Systems). Fluorescence intensity was expressed as a fluorescence index (FI) which was the mean fluorescence of the sample minus the fluorescence of the negative control divided by the mean fluorescence of the negative control.

Isolation of dendritic cells (DC) by the standard procedure

DC were isolated according to the method described by Steinman et al. (1979), with slight modifications. Spleens of B6 mice were injected with 1 ml collagenase (100 U/ml) (*Clostridium histolyticum*, type IV, Sigma Chemical, St. Louis, USA), cut into pieces and incubated at 37°C for 15 min in 1 ml collagenase (400 U/ml). A spleen cell suspension was obtained by mechanical agitation with culture medium (Iscove's medium (Flow Laboratories, Irvine, Scotland) supplemented with 15% heat-inactivated (30 min, 56°C) fetal calf serum (FCS), 100 µg/ml kanamycin, 100 IU/ml penicillin, 2 mM L-glutamine and 20 µM 2-mercaptoethanol (ME)). Spleen cells were spun on a discontinuous BSA gradient (Sigma Chemicals) of 10, 28 and 35% BSA ($\rho = 1.0310, 1.080$ and 1.099) at 4°C for 30 min at $10,000 \times g$. The interphase 10–28% BSA (low density cells) was removed and cultured for 90 min at 37°C in glass petri dishes. Non-adherent (NAD) cells were discarded and the culture medium replaced. After a further 18 h culture period at 37°C, NAD cells were separated by Fc receptor (FcR) rosetting. FcR-negative cells, DC, were isolated by centrifugation on Lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) for 20 min at $480 \times g$. Fractionation of spleen cells yielded an average recovery of 2.5% α band cells, 0.4% 18 h NAD cells and finally 0.2% DC of the total spleen cell population.

Isolation of DC by centrifugal elutriation (CE)

Cell separations were carried out under sterile conditions at 4°C and were performed as described by Figdor et al. (1984). The elutriation medium, PBS supplemented with 5 mM glucose, 0.2% BSA (Boehringer) and phenol red, pH 7.2,

290–300 mosM, was cooled by passage through melting ice and the centrifuge was cooled to 4°C. The flow rate was generated by pressurized air (1 bar) and was set and held constant with a flow controller (Brooks Instruments, Veenendaal, Netherlands). A 10 ml single cell suspension, containing $5-9 \times 10^8$ spleen cells, was loaded into the elutriation chamber at a flow rate of 12 ml/min and a rotor speed of 3200 rpm ($973 \times g$). Cells which flushed through the elutriation chamber, were collected (intro-fraction). The flow rate was increased to 18 ml/min and the cells were allowed to equilibrate in the elutriation chamber for 8 min. Then the rotor speed was decreased to 3100 rpm ($913 \times g$) and held constant for 8 min during which cells were collected (CE fraction 1). Cells were elutriated at rotor speeds of 3100 (913), 3000 (855), 2900 (799), 2800 (745), 2700 (693)/2600 (642), 2000 (380) and 0 rpm ($1 \times g$), numbered from 1–7, with intervals of 8 min ($745 \times g$, 16 min). Cells of CE fraction 5 were elutriated during two rotor speed intervals, namely at 2700 rpm ($693 \times g$) and at 2600 rpm ($642 \times g$), both held constant for 8 min. The rotor speed intervals were chosen to yield an optimal cell size distribution (Fig. 1). The cells were counted and sized by a Coulter ZF counter (Coulter Electronics, Mijdrecht, Netherlands) equipped with a C-1000 channelyzer attachment.

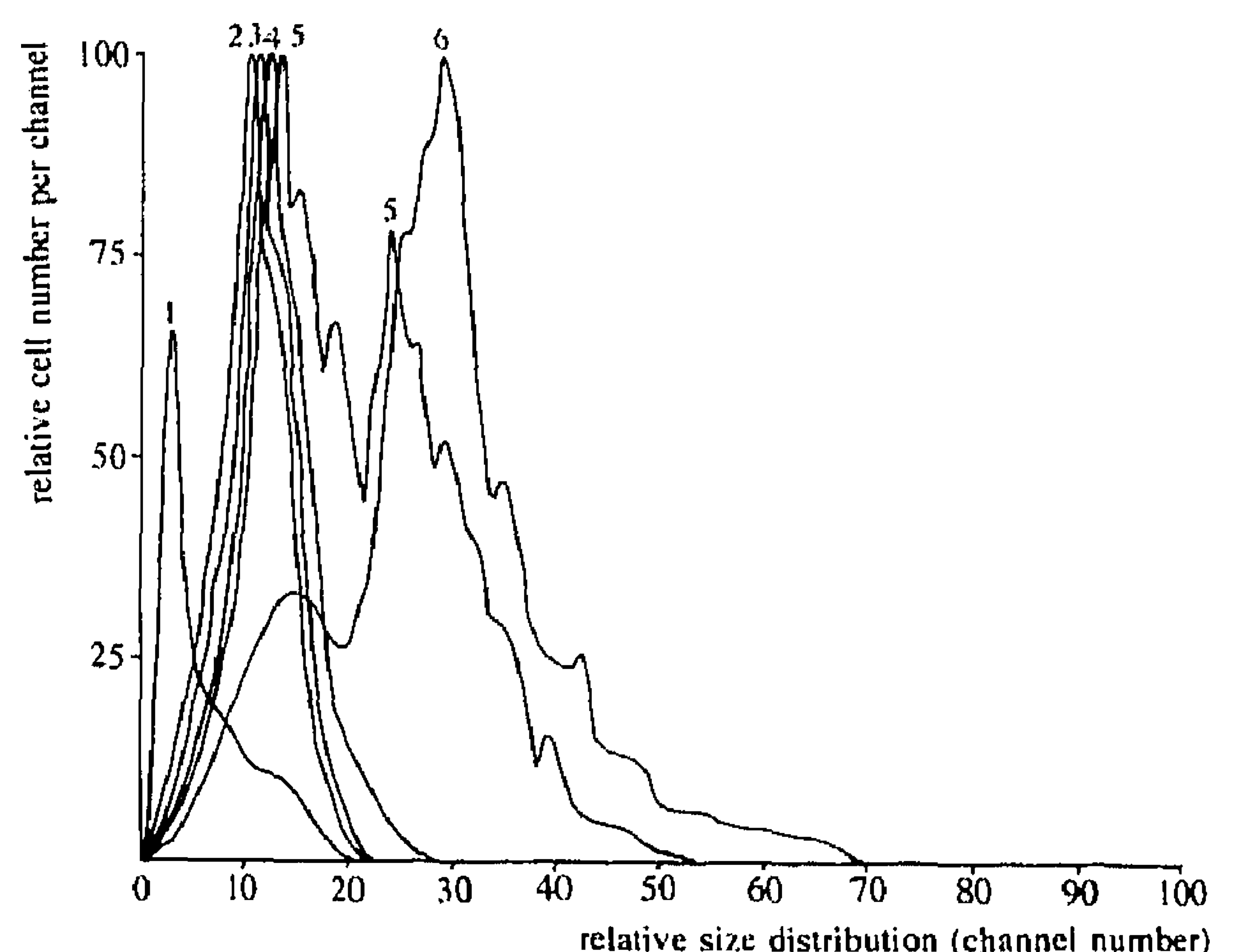


Fig. 1. Relative cell size distribution of cells from CE fractions 1–6 obtained by centrifugal elutriation. CE fraction 1 has the smallest cell size distribution and CE fraction 6 has the largest cell size distribution.

APC activity of the CE fractions

To be sure that the APC activity was derived only from the stimulator cells, responder cells were depleted of antigen presenting cells. Spleen cells from in vivo primed mice were depleted of B cells and adherent cells (APC) by a double passage through Sephadex G-10 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) (Ly et al., 1974) followed by a nylonwool passage (Scrubbed Nylon Fiber, Ferwall Laboratories, Deerfield, IL, USA) as described by Kast et al. (1988). FACS analysis showed that the responder cells contained >70% T cells (data not shown). Cell recovery after passage over all columns was approximately 13%.

The APC activities of CE fractions 1–7 were determined by a secondary Sendai virus-specific T cell proliferation assay. Various numbers of irradiated (2500 rad) cells of each CE fraction (stimulator cells) were incubated at 37°C for 60 min with 60 HAU non-virulent Sendai virus/ 10^7 cells in 100 μ l culture medium (Iscoe's medium (Flow Laboratories) supplemented with 10% FCS, 100 μ g/ml kanamycin, 100 IU/ml penicillin, 2 mM L-glutamine and 20 μ M ME) in 96 well flat-bottomed plates (Costar, Cambridge, MA, USA). Cells were washed twice by centrifugation ($270 \times g$, 4 min) after which 10^5 responder cells were added to each well. Cultures with a total volume of 200 μ l were incubated at 37°C in 5% CO₂ for 4 days, the last 4 h in the presence of 0.5 μ Ci [³H]thymidine (6.7 Ci/mmol) added in 25 μ l. The cells were harvested with an automatic cell harvester and the radioactivity was measured in a liquid scintillation counter.

Determination of the phenotype of APC in CE fraction 5

The APC activities of cells in CE fraction 5 after depletion of DC, B cells, T cells, macrophages and NK cells by treatment with a cell type specific antibody and complement was determined by a secondary Sendai virus specific T cell proliferation assay. Irradiated (2500 rad) cells in CE fraction 5 (5×10^4 cells/well) were incubated at 37°C for 30 min with saturating concentrations of 33D1, anti-B220, anti-Thy-1.2, Mac-2 or anti-asialo GM1 antibodies respectively (Table I) in 96 well flat-bottomed plates (Costar). Cells were

washed twice by centrifugation ($270 \times g$, 4 min) and were incubated at 37°C for 60 min with complement (Low-Tox-M rabbit complement, 1/12, 50 μ l/well, Cedarlane Laboratories) in culture medium supplemented with 0.5% BSA (Sigma Chemicals). Cells were washed twice by centrifugation. The complement treatment was repeated with addition of 1 μ g/ml DNase (Type 1, Sigma Chemicals) after which 10^5 responder cells were added to each well. The secondary Sendai virus specific T cell proliferation assay was continued as described above.

Further enrichment of APC from CE fraction 5

Cells in CE fraction 5 were enriched for APC by discontinuous density gradient centrifugation or depletion of B and T cells by treatment with antibody and magnetic beads. Low density cells from CE fraction 5 were isolated by centrifugation on a discontinuous gradient of BSA as described above. For the treatment with antibody and magnetic beads, cells from CE fraction 5 were incubated at 4°C for 30 min with saturating concentrations of anti-B220 and anti-Thy-1 (Table I). Cells were washed twice by centrifugation ($300 \times g$, 4 min) and were incubated at 4°C for 45 min with sheep anti-rat IgG (SAR) magnetic beads (Dynal, Oslo, Norway) (cell:magnetic bead ratio = 1:4). Cell-magnetic bead aggregates were removed by a magnet (Dynal).

Primary response

Low density cells (α band) from CE fraction 5 obtained by discontinuous density gradient centrifugation or DC isolated by the standard procedure (1.25×10^6 cells/well) were incubated at 37°C in 50 μ l 20 μ M 9 amino acid long Sendai virus nuclear protein peptide (S9: amino acid sequence 324–332) (Kast et al., 1991a; Schumacher et al., 1991) in serum-free culture medium in 96 well U bottomed plates (Costar). After 4 h of culture, 0.5×10^6 nylonwool passed spleen cells (>70% T cells) of non-immunized B6 mice in 50 μ l were added to each well. After 5 days culture at 37°C in humidified air with 5% CO₂, cells were harvested by density centrifugation on Lympholyte M (Cedarlane Laboratories). Viable cells were washed twice and used as effector cells in a cell mediated cytotoxicity assay as described by

Kast et al. (1988). In brief, various numbers of effector cells and 2×10^3 $\text{Na}_2^{51}\text{CrO}_4$ (^{51}Cr)-labeled target cells were incubated for 6 h in 100 μl culture medium in 96 well U bottomed plates (Costar). After culture, 50 μl supernatant were collected. We used EL4 target cells (thymoma cell line of B6 origin) (Gorer, 1950) resuspended in culture medium supplemented with or without 20 μM S9 or 20 μM 10 amino acid long adenovirus type 5 E1A peptide (A10: amino acid sequence 234–243) (Kast et al., 1989, 1991b) before adding them 1:1 (v/v) to the effector cells. The percentage specific ^{51}Cr release was calculated by the formula: % specific lysis = $100 \times [(\text{cpm experimental well} - \text{background } ^{51}\text{Cr release}) / (\text{cpm 2\% Triton X-100 release} - \text{background } ^{51}\text{Cr release})]$. The background release was always less than 27%. No difference in background release was observed between EL4 cells or EL4 cells sensitized with S9 or A10.

Results

Characterization of the CE fractions

Spleen cells of B6 mice were separated by CE into seven size and density dependent subpopulations. The rotor speed intervals were chosen to yield the most optimal size distribution of the cells (Fig. 1). Each successive CE fraction had a progressively larger relative cell size distribution. Cells in CE fraction 1 had the smallest size and CE fraction 6 contained the largest cells (Fig. 1). The cell size distribution of CE fraction 7 is not shown, because that fraction mainly contained B and T cells that were pelleted at the bottom of the elutriation chamber. The cell size distribution of CE fraction 7 was similar to CE fraction 3. The average recovery from the unseparated spleen cell population in the CE fractions was 75%. The percentages of recovered spleen cells of the CE fractions are shown in Table II. Almost all erythrocytes were collected in CE fraction 1 (data not shown). Subsequently, they were removed from this CE fraction by NH_4Cl treatment. The remaining leukocytes of this CE fraction and the leukocytes of CE fractions 2–6 were characterized by FACS analysis (Fig. 2). The distribution of B cells, T cells and macrophages among the

size dependent CE fractions was similar as described by Thompson et al. (1983). B cells were mainly present in CE fractions 1, 2 and 5. Most T cells were collected in CE fractions 3, 4 and 5. Macrophages were mainly present in CE fractions 5 and 6. DC, 33D1-positive cells, were present in CE fractions 4, 5, and 6. CE fraction 5 contained most of the DC (Fig. 2).

APC activity of the CE fractions

DC can be distinguished from other antigen presenting cells by their excellent APC activity as illustrated by their capacity to present Sendai virus (Kast et al., 1988). To determine which CE fraction has the highest APC activity on a per cell basis, irradiated, Sendai virus-infected cells of each CE fraction were used as stimulator cells in a secondary Sendai virus-specific T cell proliferation assay using a responder/stimulator ratio of 2:1, 6:1 and 12:1 (Fig. 3). Cells in CE fractions 1, 2 and 7 and those from CE fractions 3, 4 and 6 showed no or little antigen presenting capacity, respectively. Cells from CE fraction 5 exhibited the highest APC activity. To determine whether the excellent APC activity of cells from CE fraction 5 was mediated by DC present in this fraction, cells of CE fraction 5 were depleted of B cells, T cells, NK cells, macrophages or DC by treatment with antibody (anti-B220, anti-Thy-1.2, anti-asialo G_{M1} , Mac-2 or 33D1 respectively) and complement before use in a secondary Sendai virus-specific T cell proliferation assay. As a control, cells from CE fraction 5 were treated with complement alone. A decrease of [^3H]thymidine incorporation was observed only in mixed lymphocyte cultures stimulated with DC-depleted cells of CE fraction 5 (Fig. 4). Thus, the excellent APC activity of cells from CE fraction 5 is due to the activity of DC.

Further enrichment for DC of CE fraction 5

Recovery. FACS analysis showed that the percentage of 33D1-positive cells in CE fraction 5 was 6–10% (Table III). Further enrichment by isolation of the low density cells of CE fraction 5 by discontinuous density gradient centrifugation resulted in a mean percentage of 35–55% 33D1-positive cells. The average recovery from spleen cell input was 0.4%. Similar results were obtained

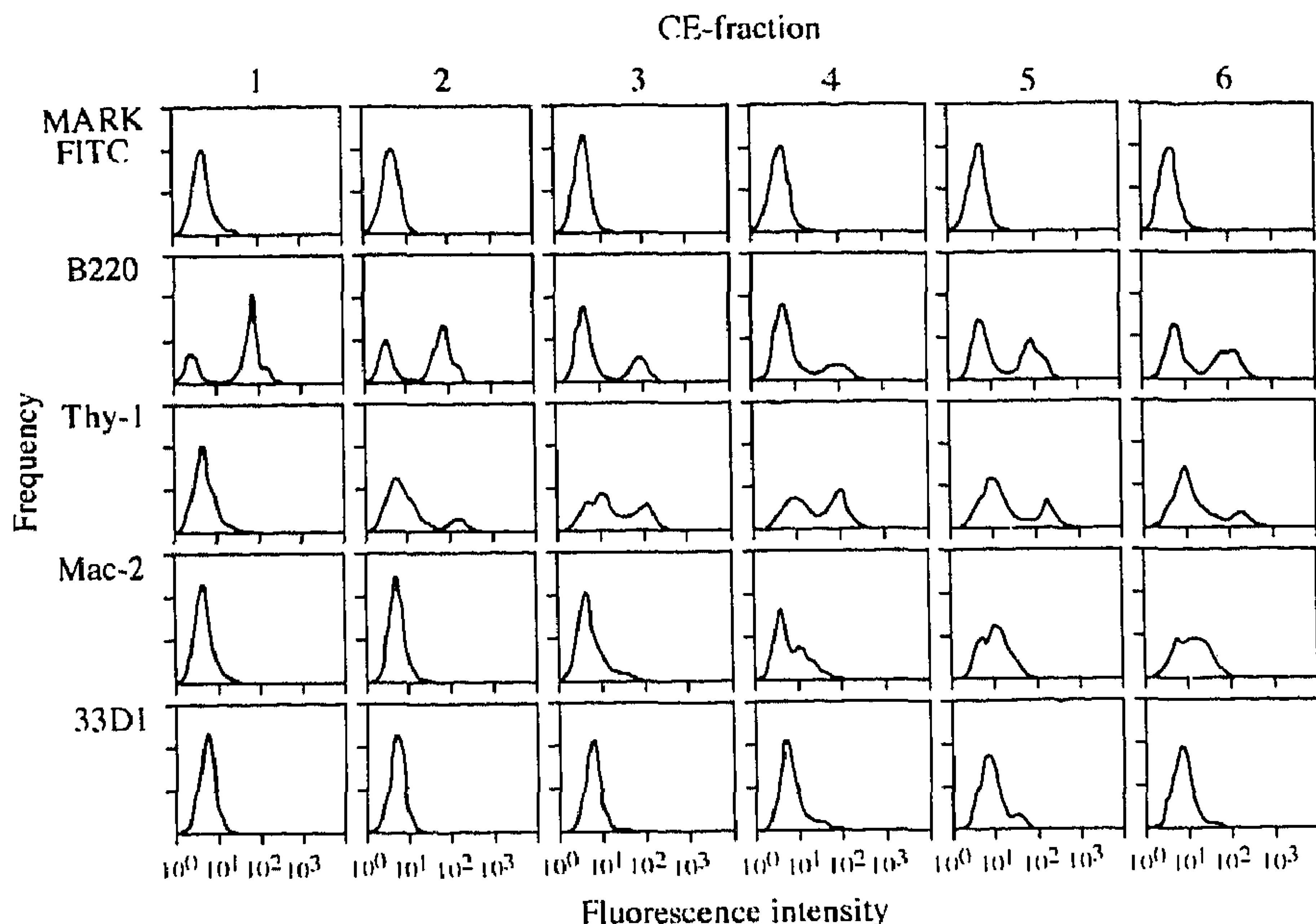


Fig. 2. Distribution of T cells, B cells, macrophages and DC among the size dependent CE fractions. Frequency versus fluorescence intensity profiles are given for CE fractions 1–6. Cells of these CE fractions were stained with different mAb and FITC-labeled mouse anti-rat Ig and analysed by FACS analysis (see materials and methods section). The MAbs are listed in Table I.

after depletion of T and B cells by treatment with antibody (anti-Thy-1.2 and anti-B220, respectively) and magnetic beads (data not shown). Although the average recovery from spleen cell input of DC isolated by the standard isolation procedure was 0.2%, the absolute number of DC isolated by both isolation procedures was similar, because the percentage of 33D1-positive cells in

TABLE II
PERCENTAGE OF RECOVERED SPLEEN CELLS IN THE SEVEN CE FRACTIONS

CE fraction number	Rotor speed (rpm ($\times g$))	% of recovered cells ($n = 8$)
Intro	3200 (973)	6.2 ± 0.7^a
1	3100 (913)	8.5 ± 1.1
2	3000 (855)	11.1 ± 1.5
3	2900 (799)	18.9 ± 1.3
4	2800 (745)	17.8 ± 1.4
5	2700 (693)/2600 (642)	15.6 ± 1.4
6	2000 (380)	13.1 ± 1.5
7	0 (1)	8.4 ± 0.8

^a Percentage \pm SE.

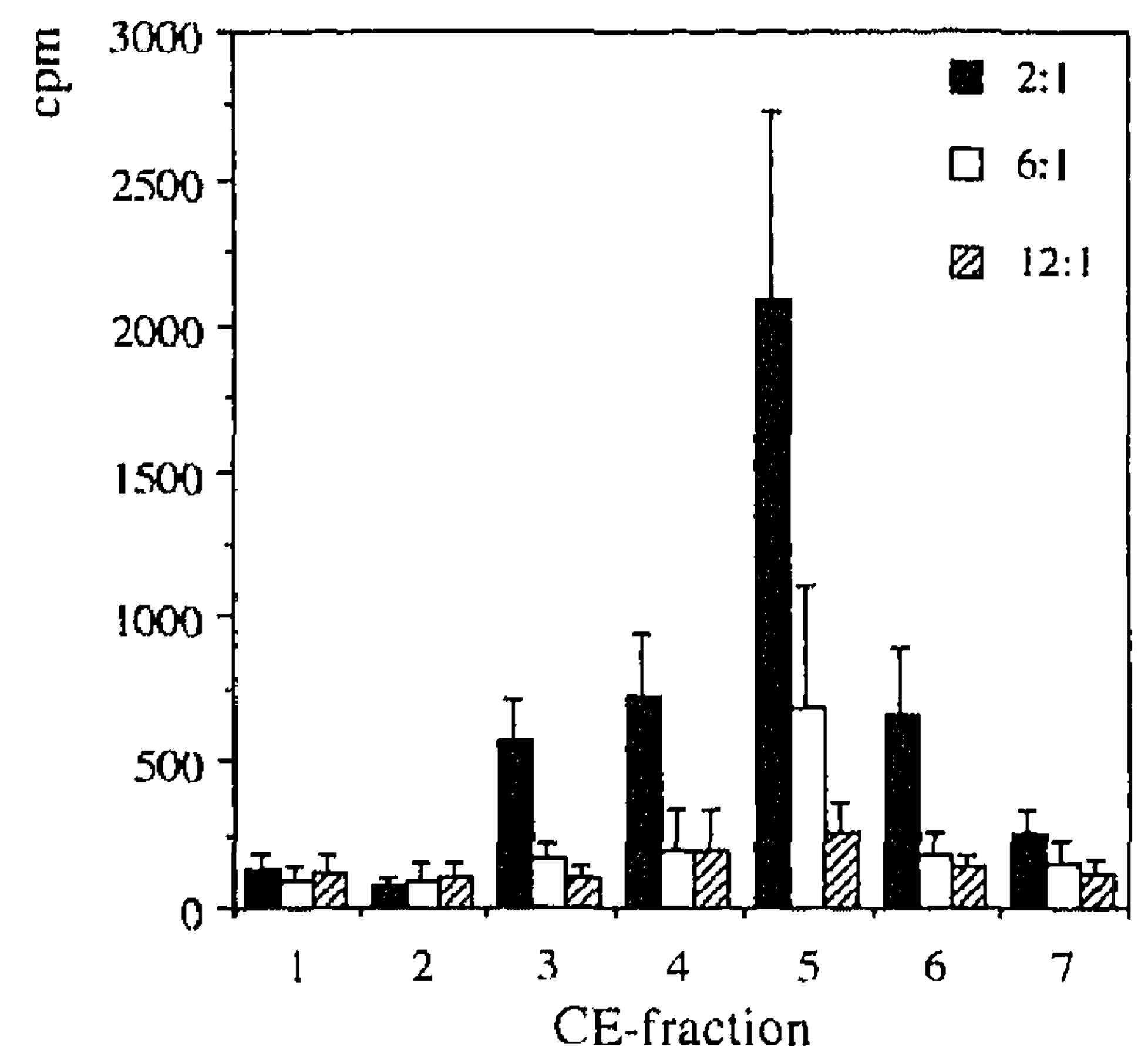


Fig. 3. APC activity of the CE fractions in a secondary T cell response. The APC activities of cells in CE fractions 1–7 were measured by a secondary Sendai virus-specific T cell proliferation assay in different responder:stimulator ratios (see materials and methods section). Data are presented as means of triplicate determinations \pm SD. [³H]thymidine incorporation of cultures stimulated with non-infected cells was less than 100 cpm. Similar results were obtained in three independent experiments.

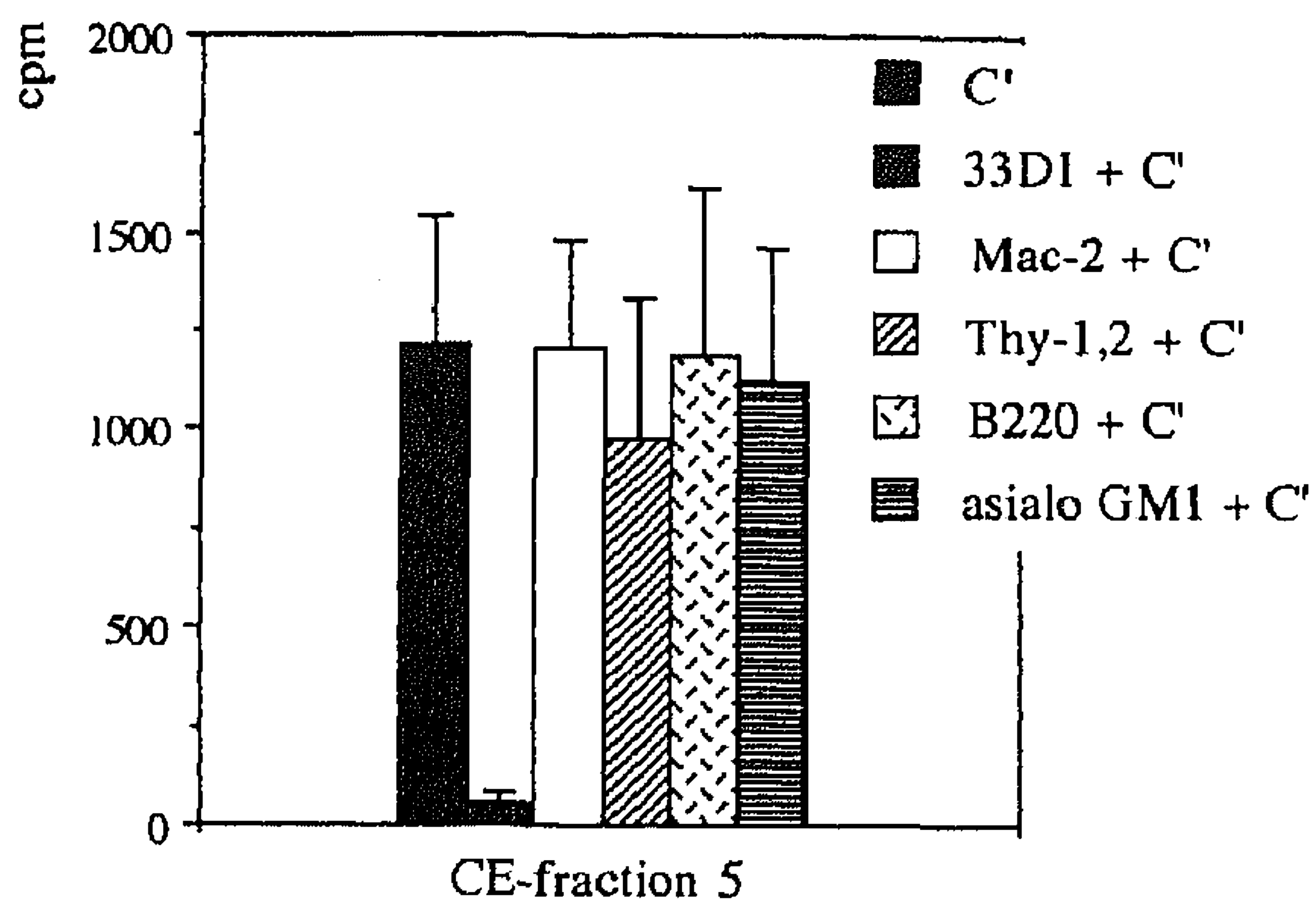


Fig. 4. Determination of the phenotype of APC in CE fraction 5. The APC activities of cells in CE fraction 5 after depletion of different cell types were measured in a Sendai virus-specific T cell proliferation assay (see materials and methods section). Cells of CE fraction 5 (5×10^4 cells/well) were depleted of DC, macrophages, T cells, B cells and NK cells by treatment with 33D1, Mac-2, anti-Thy-1.2, anti-B220 and anti-asialo G_{M1} antibodies respectively and complement after which 10^5 responder cells were added to each well. As a control, stimulator cells were treated with complement (C') alone. Data are presented as means of triplicate determinations \pm SD. The [3 H]thymidine incorporation of cultures stimulated with non-infected cells was less than 185 cpm. Similar results were obtained in three independent experiments.

TABLE III
YIELD AND PERCENTAGE OF 33D1-POSITIVE CELLS USING THE VARIOUS ISOLATION PROCEDURES

Cell fractions	33D1-positive cells	Recovery (% of spleen cell input)
CE fraction 5	6–10%	15.6 ± 1.4 ^a
Low density cells of CE fraction 5	35–55%	0.4 ± 0.07
DC isolated by the standard procedure	70–90%	0.2 ± 0.08
Unseparated spleen cells	< 1%	–

^a Percentage \pm SE ($n = 8$).

DC isolated by the standard isolation procedure was 70–90%.

APC activity in a secondary T cell response. To determine whether a further enrichment in 33D1-positive cells would result in an increase in APC activity, irradiated, Sendai virus-infected low density cells from CE fraction 5 obtained by discontinuous density gradient centrifugation, cells of CE fraction 5, DC isolated by the stan-

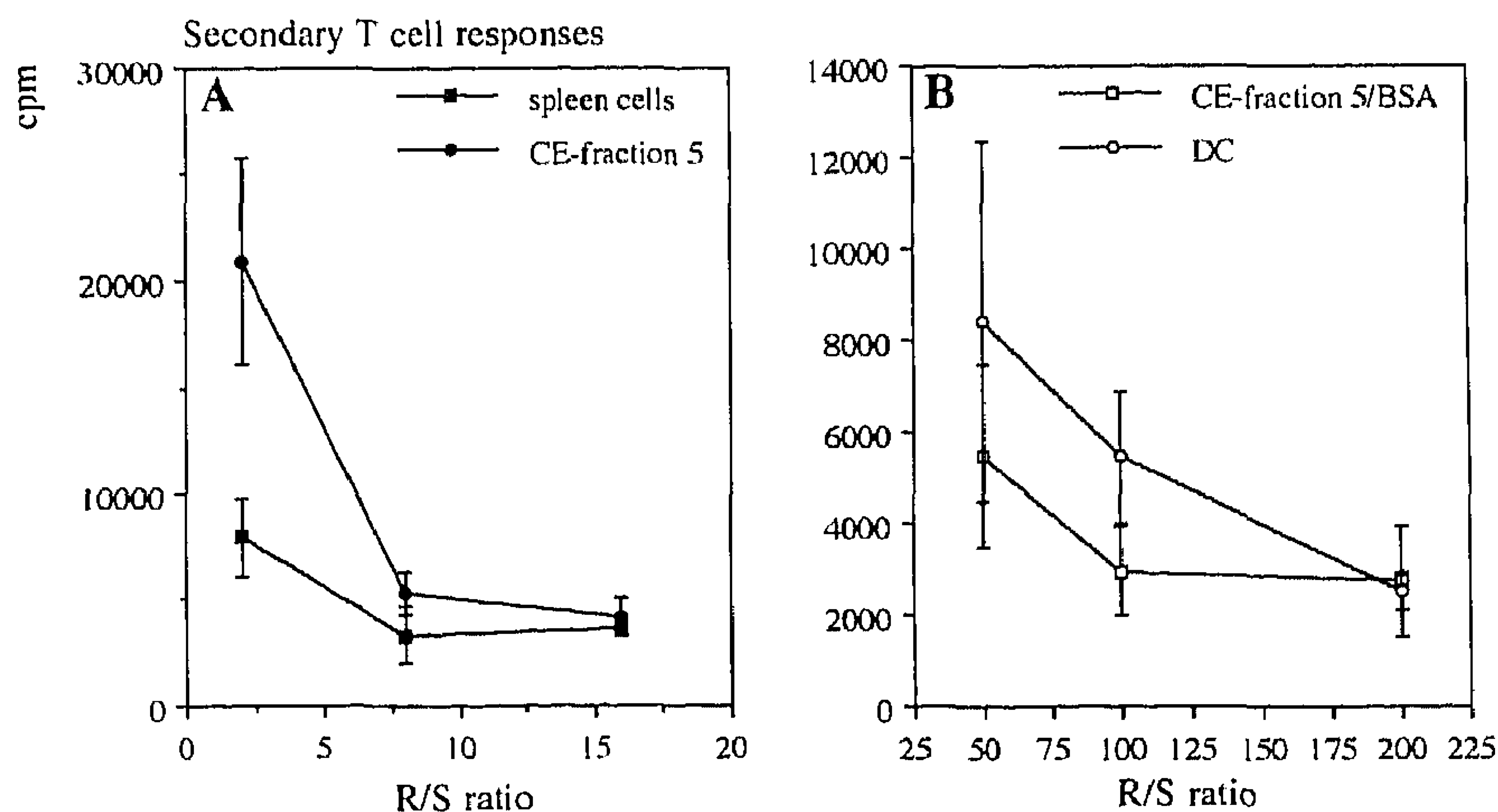


Fig. 5. APC activity in a secondary T cell response. The APC activities of non-separated spleen cells (A), cells of CE fraction 5 (A), low density cells (α band) of CE fraction 5 obtained by discontinuous density gradient centrifugation (B) and DC isolated by the standard method (B) (stimulator cells) were measured in a secondary Sendai virus-specific T cell proliferation assay at different R:S ratios (see materials and methods section). Data are presented as means of six determinations \pm SD. The [3 H]thymidine incorporation of cultures stimulated with non-infected cells was less than 1200 cpm. Similar results were obtained in three independent experiments.

standard isolation procedure and non-separated spleen cells were used in a secondary Sendai virus-specific T cell proliferation assay at different responder/stimulator ratios. Cells of CE fraction 5 had a better APC activity than unseparated spleen cells on a per cell basis (Fig. 5A). The APC activity of the low density cells of CE fraction 5 and DC isolated by the standard procedure was not significantly different, although DC isolated by the standard procedure contain two times more 33D1-positive cells than the low density cells of CE fraction 5 (Fig. 5B). The APC activity of the low density cells of CE fraction 5 and DC in a *R/S* ratio of 2:1 to 50:1 is not shown because of the high syngeneic T cell proliferation caused by dendritic cells (Nussenzweig et al., 1980). Thus, further enrichment of DC by isolation of the low density cells of CE fraction 5 resulted in an increase in APC activity comparable to the APC activity of DC isolated by the standard isolation procedure.

APC activity in a primary T cell response. DC are the only cell type described, apart from the antigen processing defective cell line RMA-S, which are capable of inducing an anti-viral or peptide specific primary T cell response (Macatonia et al., 1989; De Bruijn et al., 1991). To confirm that DC with this capacity were present in CE fraction 5, low density cells of CE fraction 5 obtained by discontinuous density gradient centrifugation and DC isolated by the standard procedure were used to induce a primary peptide-specific cytotoxic T cell response. Low density cells of CE fraction 5 loaded with 9 amino acid long Sendai virus nuclear protein peptide (S9) could induce a primary cytotoxic T cell response to the same extent as DC isolated by the standard procedure (Fig. 6). The response was specific for the inducing S9 peptide, because target cells sensitized with an unrelated peptide of adenovirus type 5 E1A (A10) were not recognized.

MHC expression and morphology. Figdor et al. (1986) have shown an increase in MHC expression on non-activated monocytes isolated by CE after incubation for 60 min at 37°C involving cell adhesion to tissue culture plates. To determine whether adherence would also result in an increase in MHC expression on DC isolated by CE, the MHC expression on these cells was mea-

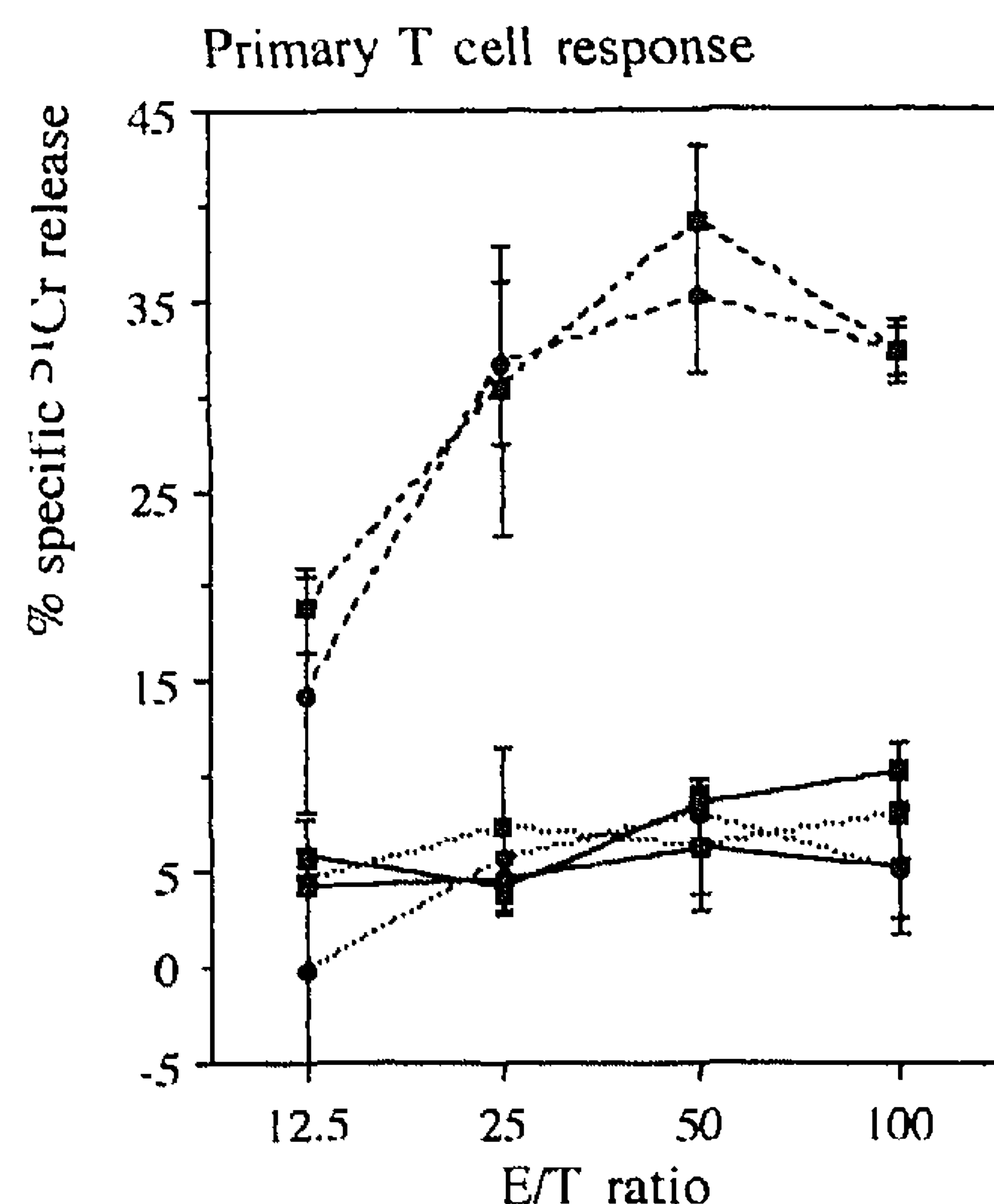


Fig. 6. APC activity in a primary T cell response. The induction of a primary CTL response in vitro with peptide-loaded low density cells of CE fraction 5 obtained by discontinuous density gradient centrifugation (■) and DC isolated by the standard procedure (●) were tested in a ^{51}Cr release assay (see materials and methods section) on the target cells: EL4 cells without (—) or with 10 μM S9 (-----) or 10 μM A10 (.....). Data are presented as means of three determinations \pm SD.

sured before and after cell adhesion. Cells of CE fraction 5 were enriched for 33D1-positive cells by depletion of T and B cells by antibody (anti-Thy-1.2 and anti-B220 respectively) and magnetic bead treatment or discontinuous density gradient centrifugation. Two-color FACS analysis showed that the mean fluorescence index of the MHC class I and class II expression on freshly isolated 33D1-positive cells of CE fraction 5 was 1.5 and 1.7 respectively (Table IV). Adherence to tissue culture plates for a period of 18 h, which is part of the standard isolation procedure, resulted in an increase of the mean fluorescence index of the MHC class I and class II expression from 1.5 and 1.7 to 5.8 and 7.7 respectively (Table IV). The mean fluorescence index of the MHC class I and class II expression on 33D1-positive cells isolated by the standard procedure was 5.1 and 4.9 respectively, comparable to 33D1-positive cells in cultured CE fraction 5 (Table IV).

None of the low density cells of CE fraction 5 had a dendritic appearance under light microscopy, although approximately 50% of these

TABLE IV
MHC EXPRESSION OF 33D1-POSITIVE CELLS ISOLATED BY THE VARIOUS PROCEDURES

	MHC expression ^a	
	Class I	Class II
33D1-positive cells of CE fraction 5: freshly isolated	1.5 ± 0.3 ^b	1.7 ± 0.7
33D1-positive cells of CE fraction 5: cultured	5.8 ± 1.8	7.7 ± 2.0
33D1-positive cells isolated by the standard procedure	5.1 ± 1.3	4.9 ± 0.6

^a DC populations were stained with 33D1-biotin and PE-streptavidin plus a FITC-labeled antibody against MHC class I (H-2D ^b, H141.31.10) or MHC class II (I-A 17-227) (see materials and methods section).

^b Fluorescence index ± SE (*n* = 5).

cells were 33D1 positive. After adherence to tissue culture plates during an 18 h culture period, approximately 50% of the cells of CE fraction 5 had a dendritic appearance. Approximately 80% of the DC isolated by the standard procedure had a dendritic appearance (data not shown). Thus, adherence to tissue culture plates resulted in an increase in the MHC expression on 33D1-positive cells and a change in their morphology.

Discussion

Dendritic cells are extremely potent antigen presenting cells, which can even restore MHC linked antigen specific and virus specific cytotoxic T cell response defects (Boog et al., 1985, 1988; Kast et al., 1988). The laborious standard isolation procedure of DC (Steinman et al., 1979) takes 2 days and may lead to the activation of these cells through adhesion to tissue culture plates. Non-activated monocytes can be isolated by centrifugal elutriation (CE) (Figdor et al., 1986; Te Velde et al., 1990). Therefore we tried to isolate DC by CE. Murine spleen cells were separated by CE into 7 size and density dependent CE fractions (Fig. 1, Table II). This procedure took 90 min. CE fraction 5 contained most of the 33D1-positive cells (33D1 = DC marker) at a con-

centration of 6–10%, which represents an approximately 15-fold enrichment compared to un-separated spleen cells (< 1% DC) (Table III). CE fraction 5 also exhibited the highest antigen presenting capacity, which fell to background levels after depletion of DC (Figs. 3 and 4). Thompson et al. (1983) have described a separation of murine spleen cells by CE in 5 fractions. The distribution of B cells, T cells and macrophages among the size dependent fractions was similar. Size-dependent B lymphocyte subpopulations have also been described by Thompson et al. (1984). They showed that resting B cells were collected in the early fractions whereas activated B cells, which expressed high levels of MHC class II, low levels of IgD and contained a high mean RNA content, were collected in the late fractions. They did not make an attempt to isolate DC. In addition none of their fractions contained cells with a high antigen presenting capacity. This was probably caused by the short time intervals used to elutriate the fractions, since this directly affects the purity of the CE fractions.

To obtain an even further enriched DC population, low density cells of CE fraction 5 were isolated by discontinuous density gradient centrifugation or cells of CE fraction 5 were depleted for B and T cells by treatment with antibody and magnetic beads, which resulted in a cell population containing 35–55% 33D1-positive cells (Table III). These two protocols were used, because they could be performed at 4°C and therefore did not allow DC to adhere to tissue culture plates. Although the isolation procedure of DC by CE is extended with a second step, the time period needed is still only 5 h versus the 2 days needed for the isolation of DC by the standard procedure.

After an 18 h culture period involving cell adhesion to tissue culture plates, the MHC expression on the 33D1-positive cells of CE fraction 5 increased to a similar level as the MHC expression on 33D1-positive cells isolated by the standard procedure (Table IV). During this same period their morphology changed from a round to a dendritic appearance. Thus, dendritic cells become activated during culture involving cell adhesion. Since DC isolated by CE from mouse spleen have a low MHC expression, they probably also

have a low MHC expression *in vivo*. DC isolated by the standard procedure are inherently activated, because this isolation procedure includes an adherence step. Despite the difference in MHC expression, the APC activity of both DC populations was similar (Figs. 5 and 6). Because, 33D1-positive cells of CE fraction 5 adhere to the tissue culture plate during the proliferation assay, their MHC expression probably rises during culture. This would explain why their APC activity is comparable to that of traditionally isolated DC. Girolomoni et al. (1990) have shown, that the MHC expression on DC isolated by cell adhesion over a period of 90 min was lower compared to DC isolated after 18 h indicating that a longer period of cell adherence results in higher MHC expression.

An alternative procedure to isolate DC without an isolation step involving cell adhesion was described by Knight et al. (1983) and Crowley et al. (1990). Knight et al. (1983) isolated DC from mouse spleen or lymph nodes by metrizamide gradient centrifugation. Metrizamide could activate DC, because Kabel et al. (1989) have shown that metrizamide apparently stimulates the differentiation of human monocytes into dendritic cells. Crowley et al. (1990) isolated DC from mouse spleen by fluorescence activated cell sorter using the antibody N418, an integrin molecule strongly expressed on DC. Although these DC were not cultured during isolation, the antibody bound to the cells may affect their function. Thus, the isolation by CE is preferable. In conclusion, DC can be isolated by CE more rapidly than will the lengthy standard isolation procedure. This new isolation procedure also avoids cell adhesion which results in activation of these cells. Therefore this new isolation procedure makes it possible to perform further studies on the activation process of DC.

Acknowledgements

This work was supported by grants from The Netherlands Organization for Scientific Research (NWO), grants 900-509-151 and 900-509-092 and by the Dutch Cancer Society grant IKW 90-23.

W.M. Kast is a fellow of the Royal Netherlands Academy of Arts and Sciences (KNAW).

We thank Drs. F. Koning, J. D'Amato and R. De Vries for critically reading the manuscript.

References

- Austyn, J.M. (1987) Lymphoid dendritic cells. *Immunology* 62, 161.
- Boog, C.J.P., Kast, W.M., Timmers, H.Th.M., Boes, J., De Waal, L.P. and Melief, C.J.M. (1985) Abolition of specific immune response defect by immunization with dendritic cells. *Nature* 318, 59.
- Boog, C.J.P., Boes, J. and Melief, C.J.M. (1988) Role of dendritic cells in the regulation of class I restricted cytotoxic T lymphocyte responses. *J. Immunol.* 140, 3331.
- Crowley, M.T., Inaba, K., Witmer-Pack, M.D., Gezelter, S. and Steinman, R.M. (1990) Use of fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J. Immunol. Methods* 133, 55.
- De Bruijn, M.L.H., Schumacher, T.N.M., Nieland, J.D., Ploegh, H.L., Kast, W.M. and Melief, C.J.M. (1991) Peptide loading of empty MHC molecules on RMA-S cells allows the induction of primary cytotoxic T lymphocyte responses. *Eur. J. Immunol.*, in press.
- De Waal, L.P., Kast, W.M., Melvold, R.W. and Melief, C.J.M. (1983) Regulation of the cytotoxic T lymphocyte response against Sendai virus analyzed with H-2 mutants. *J. Immunol.* 130, 1090.
- Figdor, C.G., Van Es, W.L., Leemans, J.M.M. and Bont, W.S. (1984) A centrifugal elutriation system of separating small numbers of cells. *J. Immunol. Meth.* 68, 37.
- Figdor, C.G., Te Velde, A.A., Leemans, J. and Bont, S.W. (1986) Differences in functional, phenotypical and physical properties of human peripheral blood monocytes (Mo) reflects their various maturation stages. In: J.J. Oppenheim and D.M. Jacobs (Eds.), *Leucocytes and Host defence*. Alan R. Liss, New York, p. 283.
- Girolomoni, G., Simon, J.C., Bergstresser, P.R. and Ponciano, Jr, D.C. (1990) Freshly isolated spleen dendritic cells and epidermal langerhans cells undergo similar phenotypic and functional changes during short term culture. *J. Immunol.* 145, 2820.
- Gorer, P.A. (1950) Studies in antibody response of mice to tumour inoculation. *Br. J. Cancer.* 4, 372.
- Ho, M.K. and Springer, T.A. (1982) Mac 2, a novel 32,000 M_R mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. *J. Immunol.* 128, 1221.
- Inaba, K., Metlay, J.P., Crowley, M.T. and Steinman, R.M. (1990) Dendritic cells pulsed with protein antigens *in vitro* can prime antigen-specific, MHC-restricted T cells *in situ*. *J. Exp. Med.* 172, 631.
- Jelachich, M.L., Lakey, E.K., Casten, L. and Pierce, S.K. (1986) Antigen presentation is a function of all B cell subpopulations separated on basis of size. *Eur. J. Immunol.* 16, 411.

- Kabel, P.J., De Haan-Meulman, M., Voorbij, H.A.M., Kleingeld, M., Knol, E.F. and Drexhage, H.A. (1989) Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator-purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation, *Immunobiol.* 179, 395.
- Kasia, M., Iwamori, M., Nagai, Y., Okumura, K. and Tada, T. (1980) A glycolipid on the surface of mouse natural killer cells. *Eur. J. Immunol.* 10, 175.
- Kast, W.M., Boog, C.J.P., Roep B.O., Voordouw, A.C. and Melief, C.J.M. (1988) Failure or success in the restoration of virus-specific cytotoxic T lymphocyte response defects by dendritic cells. *J. Immunol.* 9, 3186.
- Kast, W.M., Offringa, R., Peters, P.J., Voordouw, A.C., Meloen, R.H., Van der Eb, A.J. and Melief, C.J.M. (1989) Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. *Cell* 59, 603.
- Kast, W.M., Roux, L., Blom, H.J.J., Voordouw, A.C., Meloen, R.H., Kolakofsky, D. and Melief, C.J.M. (1991a) Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with an unbound synthetic peptide. *Proc. Natl. Acad. Sci. USA* 88, 2283.
- Kast, W.M. and Melief, C.J.M. (1991b) Fine peptide specificity of cytotoxic T lymphocytes directed against adenovirus-induced tumours and peptide-MHC-binding. *Int. J. Cancer.* 6 (suppl.), 90.
- Kelley, J.L., Rozek, M.M., Suenram, C.A. and Schwartz, C.J. (1987) Activation of human blood monocytes by adherence to tissue culture plastic surfaces. *Exp. Mol. Pathol.* 46, 266.
- King, P.D. and Katz, D.R. (1990) Mechanisms of dendritic cell function. *Immunol. Today* 11, 206.
- Knight, S.C. and Macatonia, S.E. (1988) Dendritic cells and viruses. *Immunology Lett.* 19, 177.
- Knight, S.C., Mertin, J., Stackpole, A. and Clark, J. (1983) Induction of immune responses in vivo with small numbers of veiled (dendritic) cells. *Proc. Natl. Acad. Sci. USA*, 80, 6032.
- Lake, P., Clark, E.A., Khorshidi, M. and Sunshine, G.H. (1979) Production and characterization of cytotoxic Thy-1 antibody-secreting hybrid cell lines. Detection of T cell subsets. *Eur. J. Immunology* 9, 875.
- Ledbetter, J.A. and Herzenberger, L.A. (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47, 63.
- Lemke, H., Hammerling, G.J. and Hammerling, U. (1979) Fine specificity analysis with monoclonal antibodies of antigens controlled by the major histocompatibility complex and by the Qa/TL region in mice. *Immunol. Rev.* 47, 175.
- Ly, I.A. and Mishell, R.I. (1974) Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods* 5, 239.
- Macatonia, S.E., Taylor, P.M., Knight, S.C. and Askonas, B.A. (1989) Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro. *J. Exp. Med.* 169, 1255.
- Melief, C.J.M. (1989) Dendritic cells as specialized antigen-presenting cells. *Res. Immunol.* 140, 902.
- Morse, H.C., Davidson, W.F., Yetter, R.A. and Cofman, R.L. (1982) A cell-surface antigen shared by B cells and $ly2^+$ peripheral T cells. *Cell. Immunol.* 70, 311.
- Nijhof, W. and Wierenga, P.K. (1984) Isolation of hemopoietic pluripotent stem cells from spleens of thiamphenicol-pretreated mice. *Exp. Cell Res.* 155, 583.
- Nussenzweig, M.C. and Steinman, R.M. (1980) Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* 151, 1196.
- Nussenzweig M.C., Steinman, R.M., Witmer, M.D. and Gutchinov, B. (1982) A monoclonal antibody specific for mouse dendritic cells. *Proc. Natl. Sci. USA* 79, 161.
- Ploemacher, R.E., Brons, R.H.C. and Leenen, P.J.M. (1987) Bulk enrichment of transplantable hemopoietic stem cell subsets from lipopolysaccharide-stimulated murine spleen. *Exp. Hematol.* 15, 154.
- Pretlow, T.G. and Pretlow, T.P. (1979) Centrifugal elutriation (Counterstreaming centrifugation) of cells. *Cell Biophysics* 1, 195.
- Sachs, D.H., Mayer, N. and Ozato, K. (1981) In: U. Hammerling and J.F. Kearny (Eds.), *Monoclonal Antibodies and T Cell Hybridomas*. Elsevier, Amsterdam, p. 95.
- Schumacher, T.N.M., De Bruijn, M.L.H., Vernie, L.N., Kast, W.M., Melief, C.J.M., Neefjes, J.J. and Ploegh, H.L. (1991) Peptide selection by MHC class I molecules. *Nature* 350, 703.
- Steinman, R.M., Kaplan, G., Witmer, M.D. and Cohn, Z. (1979) Identification of a novel cell-type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J. Exp. Med.* 149, 1.
- Steinman, R.M., Gutchinov, B., Witmer, M.D. and Nussenzweig, M.C. (1983) Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* 157, 613.
- Te Velde, A.A., Rousset, F., Peronne, C., De Vries, J.E. and Figdor, C.G. (1990) IFN- α and IFN- γ have different regulatory effects on IL4 induced membrane expression of Fc ϵ RIIb and release of soluble Fc ϵ RIIb by human monocytes. *J. Immunol.* 144, 3052.
- Thompson, C.B., Ryan, J.J., Siechmann, D.G., Finkelman, F.D., Mond, J.J. and Scher, I. (1983) A method for size separation of murine spleen cells using counterflow centrifugation. *J. Immunol. Methods* 63, 299.
- Thompson, C.B., Scher, I., Schaefer, M.E., Lindsten, T., Finkelman, F.D. and Mond, J.J. (1984) Size-dependent B lymphocyte subpopulations: Relationship of cell volume to surface phenotype, cell cycle, proliferative response, and requirements for antibody production to TNP-Ficoll and TNP-BA. *J. Immunol.* 133, 2333.