PRIMARY STIMULATION BY DENDRITIC CELLS INDUCES ANTIVIRAL PROLIFERATIVE AND CYTOTOXIC T CELL RESPONSES IN VITRO

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Dendritic cells (DC)[1] are potent initiators of T cell responses to alloantigens (1–4) or haptens (5–8) in vivo or in vitro using normal mice. On first stimulation in vitro, cells from normal animals show strong reactivity for these antigens by $CD4^+$, class II MHC-restricted cells. They also have a high precursor frequency of cytotoxic T cells (CTL) restricted by class I MHC antigens (e.g., reference 9), presumably selected through the antigenic history of the individual. Using infectious viruses it has not been possible so far to obtain primary T cell responses in vitro. However, DC exposed to live virus are very effective APC in vitro for secondary, antiviral responses (10, 11). In addition, Kast et al. (11) have been able to overcome CTL nonresponsiveness to Moloney virus in D^b mutant bm 14 mice by stimulating T cells from virus-primed animals with Moloney virus–infected DC in vitro. However, this protocol did not result in CTL generation to Sendai virus in bm 1 nonresponder mice.

We wished to know whether DC exposed to infectious virus were able to induce a primary, virus-specific, proliferative and CTL response. We chose the well-defined influenza system as a model for these experiments, since both Th and CTL assays and some of the viral specificities of these cells have been defined in mice (12, 13). DC were exposed to type A influenza virus or to a nucleoprotein (NP) peptide (14) recognized by BALB/c influenza-specific CTL. T cells from lymph nodes of normal specific pathogen-free (SPF) mice were stimulated with these DC in hanging drop cultures (15) that show highly efficient lymphocyte proliferation. The present study reveals that in such cultures, DC, infected with influenza in vitro or in vivo, at 0.1–1% of the number of responder T cells, resulted within 3 d in a strong proliferative response, and by 5 d in the generation of influenza-specific CTL that lysed influenza virus–infected targets. The CTL recognized the same NP peptide epitope as BALB/c CTL primed by infection (16). The NP peptide-pulsed DC also induced an antiviral CTL response. In contrast, peritoneal exudate macrophages (MO) infected with influenza did not generate a primary CTL response, although both MO and DC induced a secondary CTL response in spleen cells from mice that had been primed in vivo by influenza infection.

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[1] *Abbreviations used in this paper:* DC, dendritic cells; HAU, hemagglutination units; MO, macrophages; NP, nucleoprotein; PEC, peritoneal exudate cells; SPF, specific pathogen-free.

Materials and Methods

Antigens. Influenza virus A/X31 was grown in the allantoic cavity of 10-d-old chick embryos and stored as infectious allantoic fluid at −70°C. The influenza NP peptide 147–158 (Arg_{156}^-) has been previously described as a potent epitope for NP-specific CTL in BALB/c mice (14).

Virus Infection of Mice. 3–4-mo-old BALB/c or CBA/Ca mice were bred under SPF conditions at the National Institute for Medical Research (London). In addition to the normal mice used for primary antigen responses, mice that had been intranasally primed with A/X31 influenza virus (2–5 hemagglutination units [HAU]/mouse), 1–3 mo previously, were used for secondary antigen responses.

Responder T Cells. The lymph nodes (inguinal, axillary, and popliteal) were taken from normal SPF mice. Single cell suspensions were prepared by pressing the tissue through wire mesh and washing in medium (RPMI 1640 [Dutch modification] with 100 IU/ml of penicillin, 100 μg/ml streptomycin, 10^{-5} M 2-ME, and 10% FCS). Enriched T cells 85% pure (8) were obtained by passage of cell suspensions over nylon wool columns.

For secondary in vitro responses, T cells were purified as above from spleens of mice primed by intranasal influenza infection.

APC. DC were isolated from nonadherent spleen cells after overnight culture in medium in tissue culture flasks, or from freshly prepared lymph node cell suspensions (8, 17). The cells (5–8 ml) at 5×10^6/ml were layered onto 2-ml metrizamide gradients (Nyegaard, Oslo, Norway; analytical grade, 14.5 g added to 100 ml medium) and centrifuged at 600 *g* for 10 min. These separated cells were >80% DC, assessed by morphology and sensitivity to lysis with a specific antibody for DC (33D1; reference 18) plus C (Buxted Rabbit Co., Sussex, UK) used at a concentration of 1:10. Less than 3% were macrophages that labeled with the mAb F4/80 (19) and there were small numbers of contaminating lymphocytes (5–8).

Peritoneal exudate cells (PEC) were taken from animals 3 d after intraperitoneal injection of 60 μg Con A and were used as a source of macrophages expressing class II MHC molecules. Morphologically, 50% of the PEC were DC, which were removed by treatment with the DC-specific mAb 33D1 plus C, leaving mainly macrophages.

DC or PEC were untreated or infected in vitro in serum-free RPMI 1640 medium with influenza virus A/X31 (100 HAU/10^6 cells) for 45 min and then washed twice in medium. Alternatively, DC were pretreated at 37°C with 10^{-6} or 10^{-7} M influenza NP peptide (147–158 Arg_{156}^-) for 45 min, and washed twice in medium; some DC were treated with the contact sensitizer FITC (8) (Isomer 1, Sigma Chemical Co., St. Louis, MO) (12.5 μg/ml for 20 min at 37°C). DC exposed to antigen in vivo were isolated from the peripheral lymph nodes of mice that had been injected with 2 HAU A/X31 into each footpad and also infected intranasally with 2 HAU A/X31, 24 h previously.

Lymphocyte Proliferation In Vitro. Cultures contained $2.5–10 \times 10^4$ T cells (from normal mice or from mice primed intranasally by A/X31 infection) and varying numbers of DC or macrophages as stimulator cells, untreated or treated with influenza virus. Cultures (20 μl) were in 10-μl wells of Terasaki plates, inverted so that cells rested on the meniscus of hanging drops and were placed above sterile saline in loose-topped plastic boxes in a 37°C, well-humidified and gassed incubator (15). After 3 d, cultures were pulsed with [^{3}H]TdR (Amersham International, Amersham, UK) (2 Ci/mM, final concentration of 1 μg of thymidine) by expelling 1 μl of thymidine onto the tip of a 25-gauge needle attached to a repeating syringe (Hamilton Co., Reno, NV) and bringing this up towards the side of a hanging culture until the drop of thymidine disappeared into the culture. After 2 h the cultures were simply blotted onto 60 filter discs that had been cut in a harvester plate (Bioengineering Workshops, Clinical Research Centre, Harrow, UK) using the raised rims of a dry Terasaki plate as a cutter. Alternatively, filter discs were cut in a harvester plate using a metal cutter (Flow Laboratories, Inc., McLean, VA). The filters were washed in the harvester plate which was applied to a vacuum box attached to a tap pump. Approximately 10 ml saline and 10 ml 5% TCA were added from wash bottles and the filters were dried with alcohol (15) and counted in a scintillation counter.

Induction of Influenza Virus-specific CTL In Vitro. Purified T cells from lymph nodes of normal BALB/c mice were cultured in 20-μl hanging drops as above (10^5 T cells/droplet) in RPMI

1640 medium and stimulated with 10^3 DC infected in vitro with A/X31 influenza virus (100 HAU/ 10^6 DC). After 5 d 60 drops were pooled for the cytotoxicity assays. For secondary in vitro stimulations, splenic T cells from mice primed with infection (10^6/ml) were stimulated with DC or peritoneal exudate cells as indicated in 4 ml medium in Bijou bottles (7 ml) (Sterilin, Teddington, UK).

Cytotoxicity Assays. After 5 d, cultures in Bijoux or hanging drops were harvested and CTL activity was assayed using a ^{51}Cr release assay (12). Target cells were ^{51}Cr-labeled P815 (H-2^d) cells untreated, infected with A/X31 for 1 h, or pulsed with 10^{-7} M NP peptide (147–158 Arg_{156}^-) for 45 min at 37°C, followed by three washes before use.

After a 6-h cytotoxicity assay, percent specific ^{51}Cr release from lysed target cells, was calculated as: 100 × [cpm (sample release) − cpm (spontaneous release)]/[cpm (total release) − cpm (spontaneous release)]. Spontaneous ^{51}Cr release from targets in the absence of CTL was <10%.

Results

DC purified from spleen and infected with influenza virus in vitro stimulated a strong proliferation of T cells from normal SPF mice within 3 d, which was optimal with 1–2% DC at high concentrations of responder cells (Fig. 1 *a*). The magnitude of the response was similar to that obtained after a 2-d culture of similar T cell populations stimulated with optimal doses of the mitogen Con A (15). In contrast, untreated DC from the SPF animals did not cause significant stimulation. PEC, whether untreated or infected with influenza virus, caused no T cell proliferation (Fig. 1 *b*).

DC from lymph nodes of mice infected in vivo with influenza virus also stimulated T cells from normal CBA mice to proliferate (Fig. 2 *a*). In this figure we show that mAb HB42 to class II MHC (with Ia^K reactivity) blocked T cell stimulation by DC effectively, which suggests that at 3 d of culture, much of the T cell proliferation measured reflects class II MHC-restricted T cells. A/X31-infected PEC did not

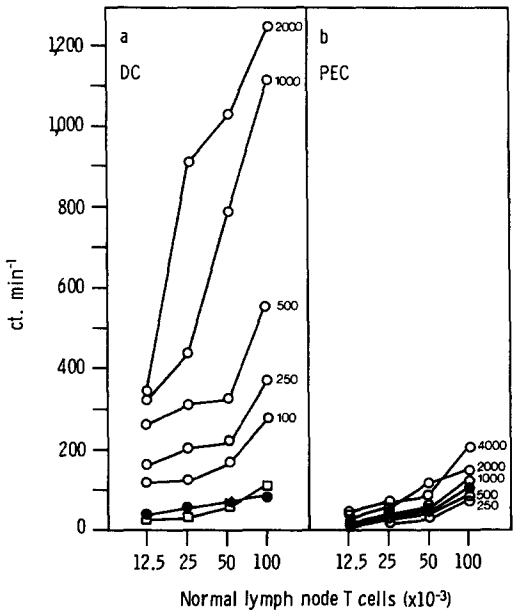

FIGURE 1. Splenic DC infected with influenza virus in vitro induce proliferation of T cells from normal BALB/c mice. Responder T cells purified from lymph nodes of normal SPF mice were cultured for 3 d with syngeneic stimulators: (*a*) 2,000 syngeneic untreated DC (□); 100–2,000 A/X31-infected DC (○); no DC (●). (*b*) 250–4,000 A/X31-infected PEC induced with Con A (○); no PEC (●).

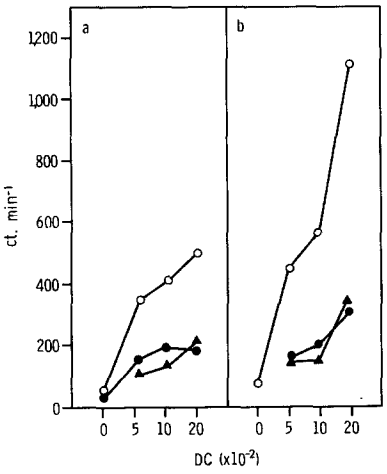

FIGURE 2. DC isolated from lymph nodes of CBA mice 24 h after influenza infection induces primary and secondary T cell proliferation. T cells (50,000) enriched from lymph nodes of (*a*) normal mice or (*b*) mice primed 2 mo previously by A/X31 intranasal infection were stimulated for 3 d with 500–2,000 syngeneic lymph node DC; normal DC (●); DC infected in vivo with A/X31 (○); or infected DC plus mAb HB42 (American Type Culture Collection, 1985; HB42 is mAb 26-8-16S selected by Sachs et al.) to class II MHC. Culture supernatant was used at 1:30 dilution (▲).

induced primary T cell proliferation, but were capable of stimulating T cells from donors primed by infection in vivo, though less efficiently than DC (Fig. 3).

We wished to examine whether DC were able to generate a primary virus-specific, CTL response. 60 replicate wells of Terasaki plates each received purified splenic DC (10^3) infected with type A influenza virus X31 and purified T cells (10^5) from lymph nodes of normal SPF mice, a combination giving high proliferative response (Fig. 1 *a*). As controls, an aliquot of the same T cells were cultured with untreated DC. After 5 d the replicate cultures were pooled and the cells tested for their capacity to lyse influenza-infected target cells. Responder T cells sensitized by influenza-infected DC, but not T cells in control cultures, strongly lysed influenza-infected P815 targets compared with very low levels of lysis of untreated target cells (Fig. 4). This CTL lysis was class I MHC restricted; P815 cells do not express class II MHC antigens and CBA (H-2^k) thioglycollate-induced macrophages after X31 in-

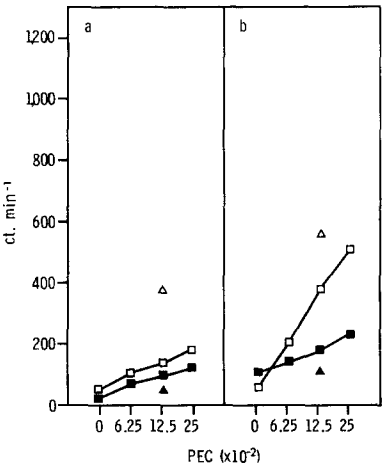

FIGURE 3. Infected PEC from BALB/c mice induce secondary but not primary T cell proliferation. Responder T cells (10^5) purified from lymph nodes of (*a*) normal mice or (*b*) mice primed 2 mo previously by A/X31 intranasal infection were stimulated for 3 d with Con A-induced purified PEC infected in vitro with A/X31 (□) or untreated (■). Responses to 1,250 A/X31-infected DC (△) or untreated DC (▲) within this experiment are shown for comparison.

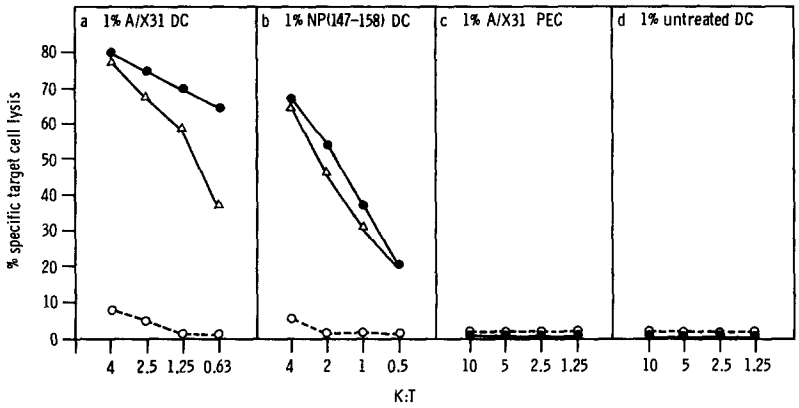

FIGURE 4. Primary induction of influenza virus-specific, and NP peptide-specific CTL by DC in vitro. 60 identical hanging drop cultures (10^5 purified responder T cells from lymph nodes of normal BALB/c mice) were stimulated for 5 d with: (*a*) DC infected with A/X31 influenza virus; (*b*) DC pulsed with NP peptide 147-158 (Arg_{156}^-); (*c*) Con A-induced PEC (after removal of DC) infected with A/X31 influenza virus; or (*d*) untreated DC. After pooling the cells in the hanging drop cultures, their cytotoxicity was assayed using a standard ^{51}Cr release assay. Target cells were ^{51}Cr-labeled P815 ($H\text{-}2^d$) cells either untreated (O), A/X31 infected (●), or pulsed with 10^{-7} M peptide 147-158 (Arg_{156}^-) for 45 min before washing (▲).

fection were not lysed (Table I). The X31-infected target cells were recognized by CBA CTL (not shown). In other experiments, cells from control cultures of T cells stimulated to proliferate with DC exposed in vitro to an irrelevant antigen (FITC) failed to lyse untreated or influenza-infected target cells (not shown). The primary BALB/c CTL generated in vitro with DC exposed to influenza virus also recognized target cells pulsed with influenza NP peptide 147-158 (Arg_{156}^-) (Fig. 4). This K^d-

TABLE I

CTL Generated by Antigen DC Are MHC Restricted

			Percent target lysis			
BALB/c CTL	Stimulation	Targets	X31	NP 147-158 (R^-)	NP 147-158	Uninfected
Normal LNT*	1% DC/NP 147-158R^-	P815	39	68	67	5
		CBA MO	0.5	-1	ND	0.6
Normal LNT*	1% DC/NP 147-158	P815	31	56	56	4
		CBA MO	-0.9	-3.8	ND	-1.2
Primed	Spleen/X31	P815	63	80	79	16
spleen cells‡		CBA MO	8	2	ND	2

Killer to target cell ratio = 10, 6-h assay.

* T cells from lymph node of normal BALB/c mice were stimulated for 5 d with DC pretreated with 10^{-6} M influenza NP peptides 147-158 or 147-159 Arg_{158}^- (R^-) in hanging drop cultures. Cytotoxic activity was tested using P815 cells or CBA thioglycollate-induced MO, untreated, infected with X31 virus, or pretreated with 10^{-7} M peptides (see Materials and Methods).

‡ Primed spleen cells were from BALB/c mice primed by intranasal infection with A/X31 virus and stimulated 5 d in vitro with A/X31-infected spleen cells.

restricted peptide is strongly recognized by CTL in BALB/c mice primed by influenza infection (14, 16). Thus, antigen presentation by influenza-infected DC in vitro for a primary CTL response mimics the response resulting from in vivo infection. PEC induced in vivo by Con A to express class II MHC molecules (P. Allen, personal communication) and infected with A/X31 influenza virus after removal of DC did not induce a primary CTL response in vitro (Fig. 4).

When responder T cells were derived from mice primed by influenza infection (12), virus-infected PEC, as well as DC, induced strong secondary CTL responses (Fig. 5), though macrophages were less efficient stimulators at the lower concentrations of APC.

In Fig. 4 and Table I we show also that a strong primary antiviral CTL response can be induced in hanging drop cultures when T cells from normal BALB/c mice are stimulated with 1% DC pretreated with 10^{-6} M NP peptide 147–158 (Arg_{156}^{-}) or the natural NP sequence 147–158. The latter peptide induced only a slightly lower CTL response than NP 147–158 (Arg_{156}^{-}). The resulting CTL strongly lysed the

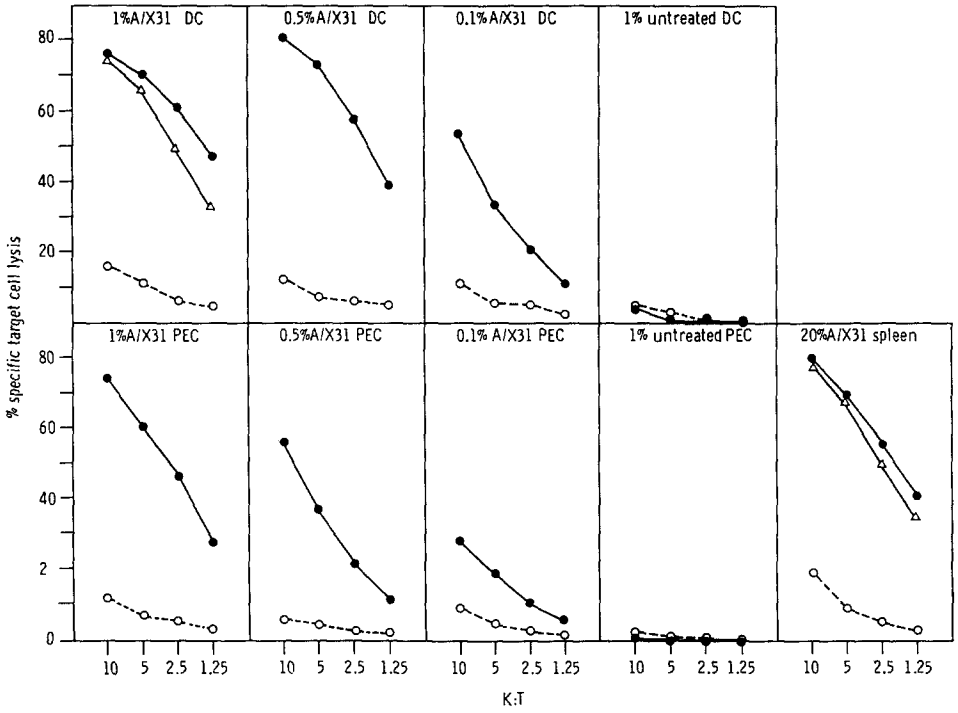

FIGURE 5. High potency of influenza-infected DC stimulator cells in generating a secondary virus-specific CTL response in vitro. Responder spleen T cells were from donor BALB/c mice primed intranasally with 4 HAU of A/X31 virus, 3 mo previously. 10^6 cells/ml were stimulated with 100–1,000 DC or Con A-induced PEC, infected with influenza virus. For comparison with our usual experimental conditions, T cells were also stimulated with A/X31-infected spleen cells (20% of responder cell number). After 5 d CTL activity was assayed using ^{51}Cr-labeled P815 target cells, either infected with A/X31 (●), pulsed with 10^{-7} M NP peptide, 147–158 (Arg_{156}^{-}) (△), or untreated (○).

P815 target cells infected with A/X31 influenza virus, or those pretreated with either of the NP peptides. The level of antiviral cytotoxicity induced by the peptide was somewhat lower than with A/X31-infected DC. The NP is a major target epitope restricted to K^d, but CTL from BALB/c mice also see other influenza proteins that are D^d or L^d restricted. We cannot exclude that some Th reactivity to the NP peptide at our high responder T cell concentration will contribute to the generation of CTL.

Discussion

DC exposed to infectious A/X31 influenza virus caused potent proliferation of T cells from normal SPF mice during a 3-d culture in as few as 12,500 lymphocytes (to a great extent, class II MHC restricted), although the virus specificity of this proliferation could only be inferred from its dependence on dose of viral antigen and increased proliferation on secondary virus stimulation. The pooling of hanging drop cultures resulted in the identification of primary influenza-specific CTL responses, although CTL precursor frequencies were previously found to be very low in our SPF mice ($\sim$1:10^6 total spleen cells or 1:3 $\times$ 10^5 spleen T cells) (20). The efficiency of the specific lysis in this system (reaching 50% with killer to target cell ratios ranging from 1 to 10 in different experiments) suggests that a very high proportion of the cells taken from the 5-d stimulated cultures were specific CTL. This could indicate that a higher proportion of T cells are able to recognize the viral peptide than previously measured, and/or that these cells are dividing very rapidly under our culture conditions. However, cells from 60 of the 20-μl cultures were pooled before the cytotoxic assay was performed so that the total initial pool of responder cells was 6 $\times$ 10^6. After 5 d of culture we recover $\sim$20% of the starting cell numbers from the pooled 60 hanging drops ($\sim 10^6$ cells) and we clearly select strongly for influenza-specific CTL that have potent cytolytic activity. Our long-term CTL clones can lyse $\sim$50% of P815 target cells at a killer to target cell ratio of 0.5. These estimates of the lytic efficiency of a single CTL may be low compared with freshly generated CTL in short-term culture. It has been shown previously that one CTL can lyse several target cells within a short time. We do not know whether occasional droplets contained a high proportion of the CTL or whether most droplets were positive. Hengel et al. (21) found that the frequency of HSV-specific CTL precursor cells in polyclonally activated T cells from normal mice was as high as 1 in 200, and as a result of this observation, they attempted to produce primary antigen-stimulated CTL directed against virus-infected targets in vitro. This was achieved in 200-μl cultures using enriched DC pulsed with high concentrations of heat-inactivated virus as stimulators, and a source of IL-2 (supernatants from Con A–stimulated spleen cells). The cells were restimulated with virus after 2 d and the culture period was extended to 7 d. The authors estimated that there was a frequency of between 1 in 500 and 1 in 10,000 HSV-specific CTL precursors in different strains of mice.

In our studies both the culture conditions and the efficiency of antigen presentation were of major importance. Primary CTL responses were not generated with the same cell combinations in similar proportions and at the same cell concentrations (5 $\times$ 10^6/ml responder cells and 1% DC) in flasks or tubes (data not shown), suggesting that the close cell/cell contacts and efficient gassing of cells in hanging drops may be essential for the observed response. Presentation of antigen by DC

was also particularly effective as shown previously for a secondary in vitro CTL response (10, 11) and by the fact that in some viral systems, and not in others, DC can restore CTL responses in nonresponder H-2 mutant mice (11).

A very recent study (22) reports that peptide-specific CTL from C57BL/6 mice can be induced in vitro by stimulation of high numbers of total spleen cells ($\sim 6 \times 10^7$/10 ml) with peptides or fragments of OVA (after cyanogen bromide treatment or trypsin treatment of OVA) and also by type A influenza NP peptide 365–380, which is recognized by D^b-restricted CTL (23). The NP peptide-specific CTL generated by Carbone et al. (22) were not obtained using influenza virus as a stimulator and did not lyse influenza virus–infected cells. We find in this study that DC pretreated with the 10^{-6}–10^{-7} M K^d restricted NP peptide 147–158 or 147–158 (Arg_{156}^-) result in the generation of CTL that also strongly lyse influenza virus–infected target cells. Using the same culture conditions described in reference 22, we could not generate BALB/c CTL specific for NP 147–158. This difference may be due to our use of DC as APC compared with peptide treatment of total spleen cells as APC for stimulation of the CTL precursor cells (22).

We observe that DC exposed to infectious influenza virus in a primary stimulation generate CTL in vitro recognizing the same NP epitope as that seen after priming of mice by infection (16). This suggests that the virus can enter the DC and that the NP would be fragmented or expressed by the DC to enable stimulation of CTL precursor cells. The very low number of macrophages (3%) present in the purified DC population was unlikely to contribute to the antigen presentation by DC exposed to infectious virus. In any case, low numbers of PEC macrophages were unable to stimulate a primary CTL response.

Our results showing the in vitro generation of CTL from T cells of normal mice by using virus-treated DC as APC also has practical implications for the study of the T cell repertoire for viral proteins in humans where the possibility of immunization with viral protein preparations is very limited. The hanging drop cultures are also effective for human lymphoid cell cultures (15). Proliferative T cell responses have already been obtained in normal human peripheral blood T cells on addition of syngeneic DC exposed to HIV in vitro (24).

Summary

We used well-gassed hanging drop (20 μl) cultures with high concentrations of purified T cells from normal BALB/c mice to examine whether dendritic cells (DC) can induce primary antiviral proliferative T cell responses and generate virus-specific CTL. We found that DC exposed to infectious influenza virus in vitro or in vivo in small numbers (0.1–1%) resulted in strong proliferation of responder T cells within 3 d, and this was strongly inhibited by antibodies to class II MHC molecules. In addition, in 5-d cultures, the influenza-treated DC generated CTL specifically able to lyse influenza-infected syngeneic target cells bearing MHC class I antigens. The most potent nucleoprotein (NP) epitope recognized by BALB/c CTL is peptide 147–158 (Arg_{156}^-) and influenza-infected DC in vitro stimulated CTL recognizing this peptide, thus mimicking the response in mice primed by intranasal influenza infection. We also induced T cell proliferation and virus-specific CTL in cultures of normal T cells by stimulating with DC pulsed with the natural NP sequence 147–158 or the potent peptide 147–158 (Arg_{156}^-). Small numbers of peritoneal exudate cells,

after activation with Con A to produce class II MHC expression and after removal of DC with a specific mAb (33DI), did not lead to primary CTL generation but initiated secondary stimulation in vitro. Our results using the hanging drop culture method and DC as APC have implications for studying the T cell repertoire for viral components in humans without the necessity of previous immunization.

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