Special Feature

The use of carboxyfluorescein diacetate succinimidyl ester to determine the site, duration and cell type responsible for antigen presentation in vivo

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Summary This report examines the use of 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to determine the site, duration and cell type responsible for antigen presentation in vivo. Evidence that CFSE-labelled T cells can be used to determine where various types of antigens are presented, including auto-antigens, oral antigens and cell-associated foreign antigens, is provided. Using this technique, the length of time antigen is presented after acquisition by APC was measured. Finally, CFSE labelling was used to identify the origin of the APC responsible for different forms of antigen presentation.

Key words: antigen presentation, CD8-positive T lymphocytes, ovalbumin, transgenic mice.

Introduction

Several years ago, we began studies to elucidate the nature of tolerance towards auto-antigens expressed in the pancreas. Early studies showed that peripheral self antigens were ignored by CD8 T cells1,2 and we have published a report consistent with this view.3 In our case, we had used the histocompatibility antigen H-2Kb (Kb) as the model auto-antigen. This antigen is recognised in its native state, but not after processing by APC. Thus, our studies did not take into account the effect of auto-antigen presentation on cells such as dendritic cells. To address this issue, we embarked on a series of new studies using ovalbumin (OVA) as a model self antigen. This approach was inspired by the ever-increasing information on the immune response to OVA and particularly by the then recent elucidation of class I-restricted epitopes of this abundant soluble protein.4 We set about generating mice expressing OVA in their pancreatic β cells, under the control of the rat insulin promoter (RIP). These were called RIP-mOVA mice, with the ‘mOVA’ referring to membrane-bound OVA.5 We also developed class I- and class II-restricted T cell receptor transgenic mice (OT-I and OT-II mice, respectively) to use as tools for detecting antigen presentation and its consequences.6,7 In our first paper examining the site of OVA presentation in RIP-mOVA mice, we used three colour flow cytometry to reveal an increase in the proportion of auto-antigen-specific transgenic T cells in those lymph nodes draining OVA-expressing tissues. We also used the rather difficult technique of bromodeoxyuridine (BrdU) incorporation to show that a large proportion of these T cells were proliferating. While such studies yielded some very interesting information, they were rather crude, required complex flow cytometry and divulged only a limited picture of the process of auto-antigen presentation. After completing the first set of experiments in this model, we became aware of a study by Hodgkin, Lee and Lyons,8 which used a technique for measuring cell proliferation developed by Weston, Lyons and Parish.9,10 This technique involved labelling of T cells with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and enabled us to track cells in vivo for their homing and subsequent proliferation in response to antigen. While our use of CFSE began some years after its initial description,9,10 we were fortunate to still be relatively early among the first wave of investigators to use this labelling technique.11 At the time, two points were clear: first, that CFSE provided a great advance over alternative techniques for the visualization of T cell activation and proliferation in vivo and second, that there would be numerous applications for this technique. In this review, we would like to describe a variety of experiments to illustrate how we have used CFSE to understand various aspects of in vivo antigen presentation.

Site of antigen presentation

We have used CFSE for identifying the anatomical site of presentation of various forms of antigen, including auto-antigen, oral antigen and foreign antigen. In addition, this technique has provided some insight into the phenotype of cells responsible for antigen presentation under these circumstances.
**Auto-antigen**

As stated earlier, RIP-mOVA mice express OVA, under the control of the rat insulin promoter, in the pancreatic β cells, the renal proximal tubular cells and at a low level in the thymus. To determine where CD8 T cells proliferate in response to OVA in these mice, CFSE-labelled OVA-specific CD8 T cells from the OT-I transgenic line were injected intravenously and then at various time points the mice were killed and their spleen and lymph nodes examined by flow cytometry. This revealed several pieces of information. First, OT-I cells only proliferated in a specific subset of lymph nodes and these corresponded to those draining OVA-expressing tissues. In these earlier studies, proliferation was seen to be restricted to the pancreatic and renal lymph nodes, but more detailed recent studies have also observed proliferation in the sacral nodes. As yet, we do not know the origin of antigen in the sacral nodes, but it is interesting to note that another line expressing secreted OVA under the control of the rat insulin promoter (RIP-OVAhi mice) also shows proliferation in the sacral lymph nodes (Fig. 1).

The second piece of information gleaned from such studies was that cell division begins about 24 h after adoptive transfer. At 25 h, the first division was evident; by 43 h, cells had progressed through a maximum of four divisions; and by 52 h, up to six divisions could be seen. Some cells had gone through more than eight divisions by 68 h. Thus, once cycling, CD8 T cells progressed at about one cycle per 5 h. This division rate, commented on in our first paper, was faster than previously expected.

The third point was that on approximately the third to fourth day, divided cells could be detected to some extent in most sites. We interpreted this to imply that divided cells had recirculated from specific sites of division to other lymphoid compartments, but we have yet to formally prove this.

**Oral antigen**

We have also been interested in responses via the oral route. Several years ago, we provided evidence that feeding large doses of OVA could lead to the induction of weak CTL responses and, under certain circumstances, to autoimmune diabetes. To examine where antigen presentation occurred after feeding OVA, we transferred CFSE-labelled, OVA-specific CD4 or CD8 T cells into normal B6 mice and then fed OVA. Two days later, we examined various lymphoid organs for the presence of dividing cells (Fig. 2, E Blanás et al. unpubl. data, 1999). This showed that OVA-specific T cells proliferated primarily in Peyer’s patches and mesenteric lymph nodes.

**Cross-priming**

A third area of interest has been the generation of CTL immunity by cross-priming. To generate OVA-specific CTL, B6 mice can be injected intravenously with irradiated spleen cells coated with whole OVA for 10 min at 37°C. In these studies, we have shown that CTL priming requires CD4 T cell help and that the CD4 T cell must see antigen on the same APC as seen by the CD8 T cell. Analysis of CFSE-labelled, OVA-specific, CD8 T cells revealed that proliferation occurred primarily in the spleen, but on day 3, divided cells could be seen to appear in the lymph nodes (Fig. 3). These latter cells are most likely the result of recirculation.

**Nature of the antigen-presenting cell**

In addition to examining the site of auto-antigen presentation in RIP-mOVA mice, we have used this approach to determine whether the cell responsible for antigen presentation is derived from the bone marrow or from parenchymal tissues. To do this, chimeric mice were generated in which OVA could be presented either only on bone marrow-derived cells or only on parenchymal cells, such as the β cells. These studies took advantage of the fact that bm1 mice, which differ from B6 mice only at the H-2K locus, are unable to present OVA to CD8 T cells. Thus, B6→bm1 RIP-mOVA chimeras only have the potential to present OVA on bone marrow-derived cells, whereas bm1→B6 RIP-mOVA chimeras may only present OVA on parenchymal cells. By examining the proliferation of CFSE-labelled OVA-specific CD8 T cells in both of these chimeras, we were able to ascertain that T cell proliferation depended on presentation by cells derived from the bone marrow (Fig. 4). We are currently using this technique to ask a similar question for the presentation of oral antigens.
antigens, which could potentially be presented by epithelial cells or conventional bone marrow-derived APC.

Duration of antigen presentation
As seen above, CFSE labelling of T cells can be very useful for identifying the site of antigen presentation and can even
help to define the APC. This approach can also be used to
determine how long antigens persist, providing a better
understanding of the kinetics of antigen presentation in vivo.

Auto-antigen persistence in cross-tolerance

In an earlier publication, we had examined the persistence of
antigen in the draining lymph node of the kidneys of RIP-
mOVA mice. To determine how long antigen persisted after
reaching the renal lymph node, we removed one kidney
and then at later time points transferred OVA-specific CD8
T cells. In our original studies, we then looked for a propor-
tional increase of these T cells in the nephrectomized renal
node relative to a non-draining node. This indicated that
antigen persisted for less than 7 days. However, in a more
recent repeat of this study using the CFSE-labelling tech-
nique, we were still able to observe proliferating cells in the
nephrectomized node on day 8 (data not shown). This experi-
ment has only been performed once and so is preliminary, but
illustrates that the CFSE labelling technique provides
increased sensitivity in this type of analysis.

Antigen persistence during cross-priming

The use of CFSE labelling has also allowed us to determine
how long antigen persists after injection of OVA-coated irra-
diated spleen cells, which normally prime CTL immunity.14
In these studies, groups of mice were injected with antigen
and then CFSE-labelled, OVA-specific CD8 T cells were
adoptively transferred on different days to detect antigen pre-
sentation. This revealed that T cells could be transferred up
to day 4 after priming and they would still begin to prolifer-
ate (Fig. 5). Any later, however, and little proliferation was
evident. Thus, antigen persists for approximately 4 days after
intravenous injection of OVA-coated irradiated spleen cells.

Islet destruction leads to cross-presentation

Besides determining the persistence of antigen presentation,
we have used CFSE to show that tissue destruction can lead
to the onset of antigen presentation in the draining nodes.12
These studies revealed that when activated CTL kill islet
β cells, antigens can be released that are then presented to
naïve CD8 T cells in the draining lymph nodes. Interestingly,
by following the activation of CFSE-labelled CD8 T cells,
we were able to show that presentation did not begin until
4–5 days after the initiation of β cell destruction. This sug-
gested that presentation under these circumstances may
require the recruitment of an APC. We have yet to devise a
strategy to formally address this issue.

Effects on the rate of T cell proliferation

Thus far, we have examined the use of CFSE labelling to
understand various aspects of antigen presentation. However,
this technique also provides a great deal of information about
the responding T cell. While we have not undertaken sophis-
ticated analysis of peak size and form, as likely discussed by
others in this issue, we have used this technique to answer
important questions about the rate of T cell proliferation
under different circumstances.

Figure 5  Presentation of ovalbumin (OVA) in the spleen per-
sists for 4 days after i.v. injection of OVA-coated irradiated spleen
cells. B6 mice were injected with 20 × 10⁶ irradiated (1000 cGy)
spleen cells previously coated with 10 mg/mL OVA for 10 min at
37°C, as previously described.16 At 2, 4 or 5 days after priming,
mice were injected with 2 × 10⁶ carboxyfluorescein diacetate
succinimidyl ester (CFSE)-labelled OT-I cells and then three
days later their spleen cells were analysed by flow cytometry.
Histograms are gated on CD8’CFSE’ cells.

Proliferation rate of autoreactive T cells

Despite the fact that autoreactive CD8 T cells proliferated in
pancreatic and renal lymph nodes of RIP-mOVA mice, these
cells were eventually deleted11 by a CD95-dependent mecha-
nism.17 In a subsequent study, we showed that co-injection of
autoreactive CD4 T cells impaired the deletion of CD8 T cells
and led to induction of autoimmunity.18 One question that
arose from these studies was whether the provision of ‘help’
enabled the CD8 T cells to proliferate more rapidly. To
address this issue, we examined the proliferation rate of
CFSE-labelled CD8 T cells, when adoptively transferred with
or without CD4 T cell help. This clearly showed that prolif-
eration was not enhanced by the provision of help.19 Further-
more, the ability to stain for CD95 on CFSE-labelled cells
revealed that this molecule was similarly up-regulated on
CD8 T cells, whether help was provided or not (data not
shown).
In another series of experiments, we showed that lack of CD30 expression by autoreactive CD8 T cells led to a dramatic increase in their numbers in vivo. In these studies, we used CFSE labelling to show that proliferation was most likely enhanced after recognition of antigen on the parenchymal tissues.

**Conclusions**

In summary, we have used CFSE labelling of T cells to detect the site of antigen presentation, the origin of the APC and the proliferation rate of T cells. This has taught us that responses to auto-antigens occur in draining lymph nodes, that oral antigens are processed mainly in the gut-associated lymphoid tissues, that i.v. injected antigens prime in the spleen and that the APC responsible for cross-priming and cross-tolerance are of bone marrow origin. The few applications for which we have used CFSE labelling have given us great insight into in vivo immunity and it is with some excitement that we look forward to the likely numerous other uses for this technique.

**References**


