T CELL-MEDIATED INHIBITION OF THE TRANSFER OF AUTOIMMUNE DIABETES IN NOD MICE

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The nonobese diabetic (NOD) mouse provides a relevant model for insulin-dependent diabetes mellitus (IDDM). Spontaneous IDDM is usually observed after 12 wk of age and largely predominates in females (1). The role of autoimmune phenomena in the destruction of islet β cells in this model is indicated by islet infiltration by mononuclear cells, mainly T lymphocytes (2, 3), by prevention of IDDM by neonatal thymectomy (4), and by in vivo treatment with cyclosporine A (5), anti-CD4+ (6, 7), or anti-I-A mAbs (8). In addition, adoptive transfer of overt diabetes has recently been obtained both in healthy NOD neonates (9) and in pre-irradiated adult male recipients (10, 11) by injection of spleen cells collected from diabetic NOD mice. The need for irradiation in order to render nondiabetic adult NOD mice susceptible to diabetes transfer appears around 5 wk of age in females and around 3 wk of age in males (9). Diabetes transfer depends in both models on the simultaneous presence of CD4+ and CD8+ cells (9, 11). The role of T cells is further underlined by the predominance of Thy-1.2+ cells in islet infiltrates (3), the absence of diabetes in NOD nu/nu mice (12), and by efficient transfer of the disease in B cell-deprived recipients (13). Finally, it is worth noting that the NOD mouse disease shows a polygenic susceptibility that includes clear genetic linkage with genes mapping within the MHC (14-17).

The role of regulatory T cells in the development of antipancreatic autoimmunity remains unclear. Induction of early diabetes in young male as well as female NOD mice by injections of cyclophosphamide (18) and the requirement for the pre-irradiation mentioned above to transfer IDDM into adult recipients (10), suggested a role of suppressive immune phenomena whose nature has remained elusive in other systems.

The adoptive transfer model provides a sensitive assay for further establishing the existence of such suppressive phenomena and studying their mechanisms. In this study, we bring direct evidence for suppressor cells that inhibit the transfer of diabetes in 8-wk-old pre-irradiated NOD recipients. We show that suppressor cells in this model are age and sex dependent, are CD4+ cells, and are abrogated by thymectomy at 3 wk of age.

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Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic mice.
Materials and Methods

Mice. NOD mice were bred in our facilities under specific pathogen-free conditions. The spontaneous incidence of diabetes in our colony reaches 38.6% in females and 0% in males by 6 mo of age. In all experiments, mice were monitored for glycosuria three times a week (Glukotest; Boehringer-Mannheim, Mannheim, FRG). Glycosuric mice and all mice at the end of each experimental procedure were checked for fasting glycemia by using test strips and a colorimetric assay (Haemoglucotest and Reflolux F; Boehringer-Mannheim). Diabetes was diagnosed when permanent fasting glycemia above 3 g/liter occurred. Hybridoma lines 4.221, 3.155, 172.4, 30-H12, 53-6.7, and GK 1.5 were grown in the form of ascites in Swiss nude mice purchased from Iffa-Credo (IARBresle, France), as previously described (9).

Adoptive Transfer of Diabetes. Recipients were preirradiated (750 rad) 8-wk-old male NOD mice, except in one experiment in which 8-wk-old female recipients were used. The transfer of diabetes was performed by injecting intravenously $10^8$ spleen cells from diabetic mice in the recipients irradiated 48 h earlier (10), the spleen cells from overtly diabetic NOD mice being prepared aseptically in HBSS and pooled before transfer.

Passive Transfer of Protective Spleen Cells. Thymic and spleen cells were collected from male and female nondiabetic NOD mice of various ages. They were prepared aseptically in HBSS and pooled within each age range or experimental group, then injected intravenously (20 x $10^6$ cells per recipient mouse) either unmodified or after lymphocyte depletions with various mAbs as described below.

Cell Fractionations. Rat mAbs used for cell depletion were obtained from clone 4.221 for Thy 1.2' cells, clone 3.155 for CD8' cells, and clone 172.4 for CD4' cells as previously described (9). Depletion of Thy-1.2' cells, CD4' cells, or CD8' cells was performed by incubating spleen cells at a density of 2 x $10^7$ cells/ml with relevant mAbs in the form of ascites diluted 1:200 in HBSS for 45 min at 4°C, then, after washing, with rabbit complement (Cedarlane Laboratories Ltd., Ontario, Canada) diluted 1:10 in HBSS for 45 min at 37°C. Treated cells were washed three times and injected into experimental animals as described above.

Monitoring of Purified Subpopulations. Purified subpopulations were phenotyped by membrane fluorescence analysis using fluorescein-conjugated Fab fragments of sheep anti-mouse Ig (Biosys, Compiègne, France) for membrane Ig' cells, and fluorescein-conjugated purified antibodies from hybridoma 30-H 12 for Thy 1.2' cells, 53-6.7 for CD8' cells, and GK 1.5 for CD4' cells. Since 30-H 12 and 53-6.7 compete respectively with 4.221 and 3.155 for immunofluorescence staining, a fluorescein-conjugated mouse anti-$\kappa$ mAb specific for rat light chain (Biosys) was used to detect cells that might have escaped lysis by complement despite fixation of IgMx mAbs 4.221 or 3.155 (9). In all depletion experiments, <1% residual cells counted among 3-4 x $10^5$ total cells were stained with either the mAb corresponding to the depleted subset or the anti-$\kappa$ light chain antibody.

Histopathology. Paraffin sections (2 $\mu$m) of bouin-fixed pancreases were stained with hematoxylin and eosin. 16-81 islets of Langerhans were examined for each pancreas specimen. Insulitis was evaluated as the percentage of islets showing a lymphoid infiltrate within or around the islets.

Thymectomy. Thymectomy was performed at weaning (3 wk of age) by aspiration under anesthesia with Nembutal.

Statistical Analysis. Statistical analyses were performed using Student's $t$ test, the $\chi^2$ test or the Dunnet test when necessary. Histological data were analyzed using the U test of Mann and Whitney.

Results

Protection Against Diabetes Transfer by Spleen Cells from Nondiabetic NOD Mice. To evaluate the protective effect of lymphoid cells from nondiabetic NOD mice against diabetes development upon transfer of the disease, irradiated NOD recipients were reconstituted with lymphoid cells collected from nondiabetic NOD donors 24 h before the transfer of diabetogenic cells collected from diabetic NOD mice. All recipient animals were indeed 8-wk-old irradiated (750 rad) NOD mice that were adop-
tively transferred 48 h after irradiation with $10^7$ diabetogenic spleen cells collected from recently diabetic NOD female mice.

As shown in Fig. 1, all control, unreconstituted animals developed overt diabetes within 4 wk after the transfer. The delay in diabetes onset upon transfer did not significantly differ when comparing female ($3.1 \pm 0.2$ wk, SE) to male ($3.1 \pm 0.4$ wk, SE) recipients. Reconstitution of preirradiated male NOD recipients with $20 \times 10^6$ spleen cells from nondiabetic 8-wk-old male or female NOD mice significantly delayed the onset of clinical diabetes after the transfer of diabetogenic spleen cells. This delay was more pronounced after reconstitution with spleen cells from female ($10.8 \pm 1.6$ wk, SE, $p < 0.001$) than male nondiabetic donors ($6.8 \pm 1.3$ wk, SE, $p < 0.01$) although male NOD mice show a lower susceptibility than female mice to spontaneous diabetes in all known colonies including our own. Importantly, spleen cells from 8-wk-old male NOD mice delayed the onset of diabetes in male recipients.

The same experimental protocol was applied to preirradiated female NOD recipients. A significant delay in diabetes onset after transfer of diabetogenic spleen cells was observed in female recipients reconstituted with spleen cells from 8-wk-old nondiabetic female ($5.2 \pm 0.7$ wk, SE, $p < 0.05$) but not male ($4.3 \pm 0.8$ wk, SE) NOD donors (Fig. 1). Noticeably, the delay in diabetes onset was of shorter duration in female than in male recipients, possibly due to higher susceptibility of female than male NOD mice to diabetes. Male 8-wk-old NOD recipients were used in all further experiments in order to avoid the possible interference of disease transferred by diabetogenic spleen cell with spontaneous disease in female recipients.

**Protection Against the Transfer of Insulitis.** Histological examination of pancreatic sections from preirradiated male recipients reconstituted with spleen cells from nondiabetic 8-wk-old female donors showed no protection against insulitis either at 9 or 16 d after the transfer of diabetogenic spleen cells (Table I). Interestingly, insulitis

![Figure 1](image-url)
**T CELL-MEDIATED INHIBITION OF DIABETES IN NOD MICE**

**TABLE I**

*Absence of Protective Activity of Spleen Cells from Nondiabetic NOD Mice against Insulitis*

<table>
<thead>
<tr>
<th>Percent of islets showing insulitis*</th>
<th>9 d after transfer</th>
<th>16 d after transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (range)</td>
<td>mean (range)</td>
</tr>
<tr>
<td>No spleen cells†</td>
<td>2.1 (0-4.2)</td>
<td>2.0 (0-6)</td>
</tr>
<tr>
<td></td>
<td>n = 2</td>
<td>n = 3</td>
</tr>
<tr>
<td>Diabetogenic spleen cells§</td>
<td>5.7 (0-12)</td>
<td>88.7 (62-100)</td>
</tr>
<tr>
<td></td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Protective§ + diabetogenic spleen cells</td>
<td>31.3 (3-80)</td>
<td>98.7 (93-100)</td>
</tr>
<tr>
<td></td>
<td>n = 4</td>
<td>n = 3</td>
</tr>
</tbody>
</table>

* Mean percent of islets showing insulitis; n indicates the number of animals in each experimental group.

† Control 8-wk-old irradiated (750 rad) male recipients that received no protective and no diabetogenic spleen cells.

§ Diabetogenic spleen cells (10⁷) collected from recently diabetic female NOD mice.

was cleared up in recipients that were not reconstituted with spleen cells from non-diabetic donors nor transferred with diabetogenic spleen cells.

**Age Dependency of the Protective Activity of Spleen Cells from Nondiabetic Mice.** A large percentage of female NOD mice, including those of our own colony, develop spontaneous diabetes after 3 mo of age, whereas males from our colony rarely show the disease and only after 6 mo of age. To evaluate the age dependency of the protective activity of spleen cells from nondiabetic female NOD mice, preirradiated male recipients were injected at 24-h intervals with 20 x 10⁶ spleen cells from nondiabetic female NOD mice of different ages, then with 10⁷ spleen cells from diabetic NOD mice. As shown in Fig. 2, spleen cells from 8-wk-old nondiabetic female donors provided the strongest protection against diabetes transfer as indicated by longest delay in diabetes onset. By contrast, spleen cells from either 2-4-d- or 26-wk-old NOD female mice provided no detectable protection and spleen cells from 2- and 5-wk-old mice only provided intermediate protective activity which, at 20 wks, was not significantly different from that of mice receiving no protective spleen cells or spleen cells from 2-4-d or 26-wk-old animals. When considering reconstitution by spleen cells from male NOD mice (Fig. 3), some protection was detected as soon as by 2-4 d of age and up to 8 wk of age as indicated by a significantly longer delay in diabetes onset upon reconstitution with corresponding cells as compared with nondiabetic spleen cells from 26-wk-old male mice.

**Protective Activity of Spleen Cells is Mediated by CD4⁺ T Cells.** To determine the role of spleen T cells in the protection provided by spleen cells from nondiabetic NOD mice, the protective effect of spleen cells depleted of whole T cells or T cell subsets from 8-wk-old female NOD mice was evaluated. As shown in Fig. 4, CD8⁺-depleted spleen cells conferred as strong a protection against diabetes transfer as total spleen cell. By contrast, Thy-1.2⁺ depleted and CD4⁺-depleted spleen cells lost any detectable protective activity against diabetes transfer, supporting the role of CD4⁺ T cells
in the protection. When considering 8-wk-old male donors the same trend was observed pointing to the role of CD4+ cells in protection although the onset of diabetes upon transfer was only very transiently delayed as previously noted with total male spleen cells (Fig. 5).

**Protective Activity of Thymic Cells.** To determine the protective capacity of thymic cells, preirradiated male recipients were transferred with $10^7$ diabetogenic spleen cells 24 h after reconstitution with thymic cells from female nondiabetic NOD donors.
Figure 4. Incidence of successful transfer of diabetes into irradiated (750 rad) 8-wk-old male recipients injected intravenously with $2 \times 10^6$ spleen cells from 8-wk-old nondiabetic NOD female donors either treated with complement alone ($\Delta$), with anti-Thy-1.2 mAb (●), anti-CD4 mAb (○), anti-CD8 mAb (▲) plus complement. A control group (●) received a reconstituted population of spleen cells obtained by added CD4⁺ and CD8⁺ spleen cells in respective proportions equivalent to that of total spleen cells from normal 8-wk-old NOD female mice. Numbers next to lines indicate the number of diabetic recipients over the total number of animals in each experimental group. The difference between groups receiving CD8⁺-depleted spleen cells, total, or reconstituted spleen cells and groups receiving either Thy-1.2 (p < 0.5 from week 11 to 17) or CD4⁺-depleted (p < 0.01 from week 11 to 17) spleen cells were statistically significant.

Figure 5. Incidence of successful transfer of diabetes into irradiated (750 rad) 8-wk-old male recipients injected intravenously with $2 \times 10^6$ spleen cells from 8-wk-old nondiabetic NOD male donors either treated with complement alone ($\Delta$), with anti-Thy-1.2 mAb (●), anti-CD4 mAb (○), anti-CD8 mAb (▲) plus complement. A control group (●) received no spleen cells from 8-wk-old nondiabetic donors. Numbers next to lines indicate the number of diabetic recipients over the total number of animals in each experimental group.

Protective capacity of spleen cells after thymectomy. To further investigate the role of the thymus in the induction of protective cells, $2 \times 10^6$ spleen cells from nondiabetic of various ages as previously reported with spleen cells. A significant protection was obtained with thymic cells from 2-4 d-, 2-wk-, and 8-wk-old mice. Some protection was still detected, although to a lesser extent, with thymic cells from 5-wk-, or 26-wk-old mice (Fig. 6). Conversely, no clear protection was observed when using thymic cells from male donors of same ages at 8 wk after transfer (Table II). The difference in the incidence of diabetes observed when comparing groups of animals reconstituted with male and female thymic cells was statistically significant at 8 wk after transfer in the 2-4-d and 8-wk groups, although not in the other age groups.
Incidence of successful transfer of diabetes into irradiated (750 rad) 8-wk-old male NOD recipients injected intravenously with $20 \times 10^6$ thymic cells collected from non-diabetic female NOD mice aged 2-4 d, 2 wk, 5 wk, 8 wk, and 26 wk 24 h before the transfer of $10^7$ diabetogenic spleen cells. A control group received no thymic cells from non-diabetic donors, although it received $10^7$ diabetogenic thymic cells 48 h after irradiation. A group was injected with spleen cells from non-diabetic male NOD mice of 2-4 d, as in Fig. 3. Numbers next to lines indicate the number of diabetic recipients over the total number of animals in each experimental group. The difference in the incidence of transferred diabetes was statistically significant when comparing recipients receiving thymic cells from non-diabetic donors aged 2-4 days, 2 wk, and 8 wk and the control group receiving no lymphoid cells ($p < 0.01$) at the end of the experiment.

### TABLE II

**Protective Activity against Diabetes Transfer of Thymic Cells from Male Nondiabetic NOD Mice**

<table>
<thead>
<tr>
<th>Age of donors of thymic cells</th>
<th>Sex of donors of thymic cells</th>
<th>Number of diabetic recipients $^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4 d</td>
<td>Male</td>
<td>0/6</td>
</tr>
<tr>
<td>2 wk</td>
<td>Male</td>
<td>1/6</td>
</tr>
<tr>
<td>5 wk</td>
<td>Male</td>
<td>5/7</td>
</tr>
<tr>
<td>8 wk</td>
<td>Male</td>
<td>1/7</td>
</tr>
<tr>
<td>26 wk</td>
<td>Male</td>
<td>5/5</td>
</tr>
<tr>
<td>No thymic cells</td>
<td>--</td>
<td>5/5</td>
</tr>
</tbody>
</table>

$^*$ $20 \times 10^6$ thymic cells were injected intravenously into preirradiated recipients.

Diabetic 8-wk-old donors that had been thymectomized or sham thymectomized at weaning were used as protector cells to reconstitute irradiated recipients. As shown in Fig. 7, thymectomy completely abrogated the capacity of spleen cells from 8-wk-old female donors to confer protection against diabetes transfer. Spleen cells from sham-thymectomized female donors showed full protective activity. Interestingly, thymectomy of male donors did not influence the protective effect of spleen cells.
Discussion

NOD mice show a high incidence of insulin-dependent diabetes that is much higher in females than in males (1). The central role of T cells in the pathogenesis of the disease is indicated by the infiltration of pancreatic β cells by T cells (2, 3), the prevention of the disease by neonatal thymectomy (4) or treatment with anti-T cell mAbs (6, 7) and the transfer of the disease into syngeneic healthy recipients by T cells obtained from diabetic mice (9-11). The absence of a pathogenic role of autoantibodies is suggested by the successful transfer of the disease into neonatal NOD mice rendered agammaglobulinemic by treatment with anti-μ antibody (13). However, the mechanism of T cell intervention in the NOD mouse is not clear. Both CD4+ and CD8+ T cell subsets are necessary to transfer the disease (9, 11). One may assume that T cells are involved at both the effector and regulatory levels but little is known of the respective role of the T cell subsets involved in cellular interactions responsible for the development of antiislet immunity. We report here data that suggest a role for T cell-mediated suppression.

The basis of our work was to obtain a model of accelerated disease by taking advantage of the low incidence of spontaneous IDDM in male NOD mice and of the high susceptibility of males to disease transfer. The transfer of diabetes in male NOD recipients was thus used as an assay system for studying protection by various lymphoid cell populations. We observed that lymphoid cells from nondiabetic NOD mice (taken before the onset of diabetes) can delay or suppress the transfer of diabetes afforded by spleen cells from overtly diabetic mice into sublethally irradiated NOD recipients. This suppressive activity was detected whenever male or female 8-wk-old recipient mice were considered although the onset of diabetes was more delayed in male than in female recipients. This may reflect the higher susceptibility to spontaneous diabetes in female than male NOD mice and simple acceleration of the disease process upon transfer of spleen cells from diabetic mice in female NOD
mice. Such higher susceptibility in female than in male recipients has been clearly evidenced upon transfer of diabetogenic spleen cells into NOD neonates (9).

The protection is mediated by CD4+ T cells since it is abrogated by in vitro treatment of spleen cells by anti-Thy-1,2 and anti-CD4 antibody plus complement but not by anti-CD8 antibody plus complement. The role of the thymic gland with regard to this suppressive activity is indicated by their absence in female mice thymectomized at weaning (3 wk of age) and by the early and potent protective activity of thymocytes. Our data indicate that the protective activity detected in spleen cells reaches its highest level at 8 wk of age in nondiabetic NOD mice. This protective activity is undetectable in spleen cells although it is detected in thymic cells in neonates. The data obtained in thymectomized mice indicate that migration of protector T cells from the thymus is probably at the origin of the suppressor CD4+ cells detected in the spleen, but it mainly occurs during the first 8 wk of life since suppression is detectable in the thymus at birth but not in the spleen until 8 wk of age. Prevention of diabetes by neonatal thymectomy is not in contradiction with our observations since it abrogates not only thymic suppressor T cells but also the generation of effector T cells responsible for the ultimate development of diabetes. The mechanism of the suppression is not clear but does not involve activation of protective cells by Y-encoded minor histocompatibility antigens expressed on male cells, since it is observed in male/male or female/female combinations. It is also unlikely that it is secondary to competition for cell homing, since when identical numbers of spleen cells are used, no protection is observed with those from 8-wk-old NOD male mice, whereas cells from 8-wk-old NOD female mice are protective. The CD4+ phenotype of the protector cells is reminiscent of previous data showing the suppressive activity of CD4+ cells (whether directly mediated by these cells or after induction of a second T cell subset, possibly CD8+), including in autoimmune diseases induced in animals against erythrocytes (19), myelin basic protein (20), or thyroglobulin (21). We recently demonstrated that injection of mAbs directed against class II MHC protects NOD mice from diabetes through the induction of CD4+ suppressor T cells (8).

In contrast with protection against clinical diabetes, no protection was observed against insulitis. Such discordance is reminiscent of the absence of a clear relation between the constant presence of insulitis in both male and female mice and the inconstant development of ultimate diabetes that predominates in female NOD mice (2, 3). Moreover, insulitis is observed as early as 4 wk of age, long before the development of hyperglycemia (2, 3). The significance of the insulitis transferred in recipients protected against clinical diabetes after reconstitution by spleen cells from nondiabetic donors was not investigated since no cellular marker is presently available to follow diabetogenic and protecting cells in recipient animals.

Indirect evidence has been interpreted as indicating that suppressor phenomena could control the development of autoimmunity in NOD mice. Irradiation of recipient NOD mice older than 3 wk of age is necessary to successfully transfer the disease by injecting spleen cells from diabetic mice (10). Cyclophosphamide induces diabetes in prediabetic NOD mice, an effect that possibly involves elimination of suppressor cells (18, 22). It is interesting to note that spleen cells from cyclophosphamide-treated mice can transfer diabetes into syngeneic preirradiated recipients,
bringing strong evidence against a direct effect of cyclophosphamide on target insulin-secreting cells (22). Last, thymectomy at weaning accelerates the onset of diabetes in female (but not in male) NOD mice (Dardenne, M., et al., manuscript in preparation). Such indirect indications that suppressor T cells are at play in the development of NOD mouse diabetes are concordant with our observation that spleen cells from non-diabetic mice can protect against diabetes transfer. One should note, however, that cyclophosphamide doses required to induce diabetes (150–200 mg × 2) (18) or irradiation doses allowing transfer of the disease (more than 550 rad) are higher than those reported to selectively abrogate suppressor T cells. It must thus be hypothesized that the effect of cyclophosphamide or irradiation in the NOD mouse model is not limited to suppressor T cells as detected in our assay.

The relatively higher suppressive activity detected in our assay system in female than in male NOD mice has to be reconciled with the higher susceptibility of female than male mice to spontaneous onset of diabetes. One possible interpretation is that the suppressive activity we report is not responsible for the lower incidence of spontaneous diabetes in male mice. The high level of protection detected in our transfer assay could be related to the high susceptibility of development of diabetes in female animals, which would lead to the hypothesis that emergence of suppressor cells in female NOD mice, although providing efficient but transient protection, is secondary to a more profound defect of physiological tolerance to islet autoantigens. Anti-β cell effector (or helper) T cells could enhance the subsequent generation of protective cells. The cellular basis of the male resistance to diabetes would then remain to be explained possibly by a less efficient effector or helper T cell function.

A key unsolved issue in this discussion is the antigen specificity of the suppressor T cells detected in female NOD mice. The question remains open due to the lack of knowledge of the β cell autoantigen. The determination of such specificity will be crucial, however, both for the understanding of the pathophysiological basis of the suppressor effect with regard to the potential therapeutic implications of such an experimental approach.

Summary

The nonobese diabetic (NOD) mouse has recently been introduced as a model for insulin-dependent diabetes mellitus. The role of regulatory T cells in the development of antipancreatic autoimmunity in this model remains unclear. To evaluate the presence of suppressive phenomena, we used disease transfer by spleen cells from diabetic NOD mice into preirradiated adult recipients as a model for accelerated disease. Suppressor phenomena were detected by testing the protection afforded by lymphoid cells from non-diabetic NOD mice against diabetes transfer in irradiated recipients. Transfer of diabetes was delayed by reconstituting recipients with spleen cells from non-diabetic NOD donors. The greatest protection against diabetes transfer was conferred by spleen cells from 8-wk-old non-diabetic female NOD mice. Depletion experiments showed that the protection was dependent on CD4⁺ cells. Protection was also detected within thymic cells from non-diabetic NOD mice and protection conferred by spleen cells was abrogated by thymectomy of non-diabetic female, but not male, NOD donors at 3 wk of age. These findings indicate that suppressive CD4⁺ T cells that are dependent on the presence of the thymus may delay the onset of diabetes in female diabetes-prone NOD mice.
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References