Use of fine needle aspirates and flow cytometry for the diagnosis, classification, and immunophenotyping of canine lymphomas

Mahmut Sözmen, Silvia Tasca, Erika Carli, Davide De Lorenzi, Tommaso Furlanello, Marco Caldin

Abstract. Fifty canine lymphomas were classified cytomorphologically using the updated Kiel classification scheme. Aspirates of lymph nodes from dogs with lymphoma were stained using 5 canine-specific antibodies and 3 human-specific antibodies that cross-react with canine lymphocytes. The antibody-stained aspirates were analyzed by flow cytometry. A total of 32 (64%) of the 50 lymphomas were characterized as B-cell origin and 18 (36%) were of T-cell origin. B-cell lymphomas were identified in 12 females and 20 males with a mean age of 8.35 years. T-cell lymphomas were identified in 8 females and 10 males with a mean age of 7.9 years. A minority of the lymphomas were low-grade B-cell and T-cell lymphomas (6/50, 12% and 4/50, 8%, respectively). The most common morphologic types were high-grade centroblastic and unclassifiable plasmacytoid for B- and T-cell lymphomas (18/50, 36% and 7/50, 14%, respectively).

Key words: Canines; flow cytometry; immunophenotype; lymphoma.

Introduction

Lymphoma is the most common hematological malignancy in dogs. The incidence of lymphoma in dogs ranges between 13 and 33 cases per 100,000 dogs and is greater than in humans. Recent studies reveal the importance of fine needle aspiration in the diagnosis of non-Hodgkin’s lymphoma/leukemia (NHL), particularly when combined with immunophenotyping by flow cytometry (FC) in both dogs and humans. Flow cytometry is routinely used in human medicine as the reference method for the immunophenotypic analysis of acute leukemia and lymphoma. However, the method has been used only rarely for the immunological classification of hematopoietic neoplasia in dogs. Cytological examination is commonly used in the diagnosis of canine lymphoma because it is considered a simple, safe, and rewarding diagnostic method. The definition of the immunophenotype of the lymphoma is important for prognosis and for development of clinical treatment protocols. The aim of this study was to investigate lymphoproliferative lesions in dogs by combining cytomorphology and FC.

Materials and methods

Animals. Fifty cases of canine lymphoid neoplasms referred to the laboratory of Clinica Veterinaria San Marco, Padua, Italy, between 2002 and 2004, were studied by immunophenotyping. None of the dogs had been treated before the analysis. Diagnosis was based on clinical, cytologic, and follow-up data. Fine needle aspirates (FNAs) were taken from palpable lymph nodes (submandibular, prescapular, popliteal), and cytological diagnosis was based on air-dried smears stained with May-GruÈnwald-Giemsa (MGG). The morphological classification criteria were based on the cell size (“small,” “medium,” or “large,” i.e., nuclei smaller than, equal to, or larger than 2 red blood cells); the shape of the nuclei; the density of the chromatin; the number, size, and distribution of the nucleoli; and the volume and basophilia of the cytoplasm. The mitotic index was estimated in cytological specimens by scanning 5 fields at 500, counting the mitotic figures, and calculating the median number. A low mitotic index was defined as 0–1 mitosis per 5 fields, medium as 2–4 mitosis per 5 fields, and high as 5 or more mitosis per 5 fields.

Staining of surface antigens. For the staining of surface antigens, 100 µl of the sample suspension were mixed with 5–10 µl (recommended volume) of each antibody in a plastic tube and incubated in the dark at room temperature for 30 min. The specificity, designation, and reactivity of the antibodies used are listed in Table 1. To lyse the erythrocytes and to fix leukocytes, 2 ml of lysing solution were diluted 1:10 in distilled water, added to the cells, and left for 15 min at room temperature. Afterward, the cells were centrifuged for 10 min at 1,200 × g, and the supernatant was aspirated with a pipette and discarded. The cells were resuspended in 1 ml of phosphate-buffered saline (PBS) before FC analysis.

Staining of cytoplasmic antigens. For the staining of cy-
Table 1. Characteristics of antibodies used.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Designation</th>
<th>Isotype</th>
<th>Conjugation</th>
<th>Leukocyte subpopulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine CD3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA1774F</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>T-lymphocytes</td>
</tr>
<tr>
<td>Canine CD4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA1038F</td>
<td>Rat IgG2a</td>
<td>FITC</td>
<td>T-helper cells, neutrophils, monocytes</td>
</tr>
<tr>
<td>Canine CD8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA1039PE</td>
<td>Rat IgG1</td>
<td>PE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>Canine CD45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA1042PA</td>
<td>Rat IgG2b</td>
<td>PE</td>
<td>Leucocytes</td>
</tr>
<tr>
<td>Human CD14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA1568C</td>
<td>Mouse IgG1a</td>
<td>PE-CY5</td>
<td>Monocytes, leucocytes</td>
</tr>
<tr>
<td>Canine CD34&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1H6</td>
<td>Mouse IgG1a</td>
<td>PE</td>
<td>Blasts</td>
</tr>
<tr>
<td>Human CD21&lt;sup&gt;e&lt;/sup&gt;</td>
<td>B-ly4</td>
<td>Mouse IgG1a</td>
<td>PE</td>
<td>B-lymphocytes</td>
</tr>
<tr>
<td>Human CD79&lt;sub&gt;a&lt;/sub&gt;cy&lt;sup&gt;e&lt;/sup&gt;</td>
<td>HM57</td>
<td>Mouse IgG1a</td>
<td>PE</td>
<td>Pan-B</td>
</tr>
</tbody>
</table>

toplasmic antigens, the Leucoperm<sup>e</sup> reagent<sup>b</sup> system for fixing and permeabilizing cells in suspension was used. This procedure gives antibodies access to intracellular structures and leaves the morphological characteristics of the cells intact. For each sample, 50 μl of the sample suspension was added to 100 μl of Reagent A (fixation medium). Five milliliters of PBS was added after 15 min of incubation at room temperature, and the mixture was centrifuged for 5 min at 1,200 × g. Supernatant was removed, and 100 μl of Reagent B (permeabilization medium) was added to the cell pellet along with 10 μl of the appropriate antibody directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-CY5 (PE-CY5) (Table 1). The sample was vortexed at low speed for 1–2 sec and incubated for 30 min at room temperature in the dark. After incubation, cells were resuspended in 4 ml of PBS and centrifuged for 10 min at 1,200 × g. Supernatant was aspirated with a pipette and discarded. The sample was resuspended in 1 ml of PBS before FC analysis.

Flow cytometric analysis and evaluation of measured data. Flow cytometry was performed with the Epics XL-MCL flow cytometer. Measured data was evaluated with the computer program System II<sup>®</sup> (DOS 6.22) Couter<sup>®</sup>. A lymphocyte gate was established using forward scatter and side scatter (SC), 2 parameters that take into account cell dimensions and cytoplasm complexity. Within the gate, percentages of subpopulations were calculated by establishing parameters for SC and fluorescence (FITC, PE, or PE-CY5). In evaluating the ensuing measurements, every cell with a higher mark was regarded as positive for the respective antibody. Lymphoma cells reacting with the antibody against CD79<sub>a</sub>cy but negative for CD3 were allocated to the B-cell line. Alternatively, lymphoma cells positive for CD3 and negative for CD79<sub>a</sub>cy were classified as T-cell lymphomas.

Results

Immunophenotyping and cytology

The results obtained with the updated Kiel classification adapted to the canine species are shown in Table 2. The main cytologic and immunophenotypic features of each type were as follows.

Table 2. Classification of 50 canine lymphomas according to the updated Kiel classification.

<table>
<thead>
<tr>
<th>B-cell lymphoma type</th>
<th>Number of cases, (32, 64%)</th>
<th>T-cell lymphoma type</th>
<th>Number of cases, (18, 36%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-grade lymphomas</td>
<td>6, 12%</td>
<td>Low-grade lymphomas</td>
<td>4, 8%</td>
</tr>
<tr>
<td>Small cell</td>
<td></td>
<td>Small cell</td>
<td></td>
</tr>
<tr>
<td>Lymphocytic</td>
<td>1, 2%</td>
<td>Clear cell</td>
<td>2, 4%</td>
</tr>
<tr>
<td>Lymphoblasticplasmacytic</td>
<td></td>
<td>Prolymphocytic</td>
<td></td>
</tr>
<tr>
<td>Prolymphocytic</td>
<td>1, 2%</td>
<td>Pleomorphic small cell</td>
<td>1, 2%</td>
</tr>
<tr>
<td>Centrocytic</td>
<td></td>
<td>Mycosis fungoides</td>
<td>1, 2%</td>
</tr>
<tr>
<td>Centroblastic-centricrocytic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macronucleated medium-sized cell*</td>
<td>4, 8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-grade lymphomas</td>
<td>26, 52%</td>
<td>High-grade lymphomas</td>
<td>14, 28%</td>
</tr>
<tr>
<td>Centroblastic</td>
<td></td>
<td>Pleomorphic, mixed, small and large cell</td>
<td>5, 10%</td>
</tr>
<tr>
<td>Monomorphic</td>
<td>1, 2%</td>
<td>Pleomorphic large cell</td>
<td></td>
</tr>
<tr>
<td>Polymorphic</td>
<td></td>
<td>Immunoblastic</td>
<td></td>
</tr>
<tr>
<td>Predominantly small cell</td>
<td>13, 26%</td>
<td>Unclassifiable, plasmacytoid*</td>
<td>7, 14%</td>
</tr>
<tr>
<td>Predominantly large cell</td>
<td>4, 8%</td>
<td>Lymphoblastic</td>
<td>2, 4%</td>
</tr>
<tr>
<td>Immunoblastic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small cell, unclassifiable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkitt-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmacytoid</td>
<td>4, 8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>4, 8%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Canine lymphomas without established human counterparts in updated Kiel classification.
B-cell lymphomas

A total of 32 (64%) lymphomas were diagnosed as B-cell type. Of these, 6 (12%) were low-grade lymphomas and 26 (52%) were high-grade lymphomas. B-cell phenotype was established in all the macronucleolated medium-sized lymphomas and in both small lymphocytic and prolymphocytic lymphomas. High-grade B-cell lymphomas included 18 (36%) cases of all the variants of centroblastic lymphomas, 4 (8%) lymphoblastic lymphomas, and 4 (8%) small cell lymphomas with plasmacytoid appearance.

Small lymphocytic cell type. The single case had a small, round nucleus with coarse chromatin, indistinct nucleolus, and scant basophilic cytoplasm. The mitotic index was low (Fig. 1).

Small prolymphocytic-like cell type. The single case was characterized by small cell size with a nucleus with fine or irregularly condensed chromatin and a prominent central nucleolus (Fig. 2).

Macronucleolated medium-sized cell type. Four cases revealed macronucleolated medium-sized cells (MMCs) with a large, prominent central nucleolus, fine chromatin, and a narrow, moderately basophilic cytoplasmic rim (Fig. 3).

Centroblastic type. Centroblastic lymphomas were the most common morphologic type B-cell lymphomas, representing more than half of all B-cell type lymphomas. They were subdivided into 2 subtypes: 1) a monomorphic subtype composed of more than 60% centroblasts, which are large cells with scant basophilic cytoplasm, a round nucleus, fine chromatin pattern, and 2–4 basophilic prominent nucleoli located in the margin (Fig. 4), and 2) a polymorphic subtype, which was further subdivided into 2 subtypes, 1 with predominantly small cells (Fig. 5) constituted about 40% (13/32) of the B-cell lymphomas and the other subtype was a predominantly large cell subtype (4/32) (Fig. 6). The small cell polymorphic type consisted mainly (up to 80%) of cells containing 2–5 small basophilic nucleoli and irregular marginal and perinucleolar chromatin stippling. Cytoplasm was scant, moderately basophilic, and forming a narrow cytoplasmic rim.

Small cell, unclassifiable plasmacytoid type. Four of the lymphomas revealed small to medium-sized cells with eccentric nuclei and moderate to abundant

Figure 1. Lymphocytic lymphoma (B cell) composed of small cells with round nuclei with coarsely clumped nuclear chromatin without nucleolus and basophilic scant cytoplasm. MGG; Bar = 5 µm.

Figure 2. Prolymphocytic-like lymphoma (B cell). Small cells with condensed chromatin, distinct central nucleolus, and slightly extended pale cytoplasm. MGG; Bar = 5 µm.

Figure 3. Macronucleolated medium-sized cell lymphoma (B cell). Homogenous medium-sized cells with fine chromatin, perinuclear thickening, prominent central nucleolus (arrow), and a narrow, moderately basophilic cytoplasmic rim. MGG; Bar = 5 µm.

Figure 4. Centroblastic, monomorphic lymphoma (B cell). Homogenous, dense population of large cells with round nucleus and several small and prominent nucleoli (arrows) and the ring of clearly basophilic cytoplasm. MGG; Bar = 5 µm.
strongly basophilic cytoplasm often with evidence of a clear Golgi zone (Fig. 7).

**Lymphoblastic type.** Four lymphomas in this phenotype were characterized by their small size and a nucleus with irregularly condensed chromatin with perinucleolar thickening and 1 to several nucleoli. Cytoplasm was scant, moderately basophilic, and formed a thin rim around the nucleus (Fig. 8).

**T cell lymphomas**

Eighteen (36%) lymphomas were diagnosed as T-cell type. Of the 7 cases that were evaluated for caCD4 and caCD8 expression, 2 were caCD4+ caCD8− and 5 were caCD4− caCD8+. Four (8%) of these lymphomas were classified as low-grade and 14 (28%) as high-grade lymphomas. The low-grade T-cell lymphomas consisted of 2 (4%) clear cell, 1 pleomorphic small cell lymphoma, and 1 mycosis fungoides types. Fourteen (28%) high-grade T-cell lymphomas included 2 (4%) lymphoblastic lymphomas, 5 (10%) pleomorphic, mixed, small and large cell lymphomas, and 7 (14%) high-grade, unclassifiable lymphomas with plasmacytoid appearance.

**Small clear cell type.** Two cases were composed of small monomorphous cells with pale cytoplasm. The nuclear surface was round to irregularly round with small nucleoli and more or less clumped chromatin (Fig. 9). The mitotic index was low. One of the 2 was evaluated for caCD4/caCD8 immunophenotype and was found to be caCD4− caCD8−.

**Pleomorphic small cell type.** A single case consisted of a population of small cells characterized by a smooth surface on one side and serrations on the opposite side with fine or more or less clumped chromatin and scant to moderate, sometimes unipolar pale cytoplasm (Fig. 10).

**Mycosis fungoides.** One case was characterized by both lymph node and cutaneous infiltration. Cells showed round or slightly irregular nuclei with fine chromatin and moderate to abundant pale cytoplasm (Fig. 11). The mitotic index was low.

**Pleomorphic, mixed, small and large cell type.** Five lymphomas were composed of atypical, small, medium, and large cells displaying considerable nuclear pleomorphism. Nuclei were often convex and smooth
on 1 side, whereas the opposite side was concave with irregular indentations. Cytoplasm was moderately abundant and lightly basophilic (Fig. 12). The single case evaluated for caCD4/caCD8 immunophenotype was caCD4 caCD8+.

Unclassifiable plasmacytoid type. Seven tumors were characterized by a proliferation of medium-sized cells with fewer small and large cells in varying proportion. These cells exhibited round or slightly irregular eccentrically located nuclei, 1 large centrally placed or multiple nucleoli, and relatively abundant, strongly basophilic cytoplasm, occasional bi- or multinuclear cells; perinuclear clear zone provided a plasmacytoid appearance (Fig. 13). Chromatin was irregularly clumped in small cells, clumped around the nucleoli in medium-sized cells, and finely dispersed in larger cells. Four cases were evaluated for caCD4/caCD8. Three of these were caCD4+ caCD8− and the other was caCD4− caCD8+.

Lymphoblastic type. Two cases contained monomorphic small to medium-sized cells, with irregularly round or slightly convoluted nuclei, finely dispersed dusty chromatin, multiple, prominent nucleoli, and scant basophilic cytoplasm (Fig. 14). The single case evaluated for caCD4 and caCD8 was caCD4+ caCD8−.

Epidemiological and biological features

Twenty of the 50 dogs with malignant lymphoma were female and 30 were male. Ages ranged between 2 and 15.5 years with an average age of 8 years. T-cell lymphomas occurred in dogs aged from 3 to 14 (mean 7.9) years, whereas B-cell lymphomas occurred in those aged 2–15.5 (mean 8.35) years. There was no sex predilection for T cell lymphomas. However, males were more often affected (20/32, 62.5%) than females with B-cell lymphomas. Sixteen different breeds were represented. An apparent breed predilection was found only in Boxers (9/50, 18%).

Discussion

In veterinary medicine, cytology has been generally accepted as a reliable diagnostic technique for canine lymphomas. Lymphoma immunophenotype classification is important in predicting biological behavior.
and response to therapy. In this study, the updated Kiel classification, which places major emphasis on cytology, was adapted and used in the canine species. Lymphomas of this study were generally classified as high-grade tumors (40/50, 80%); few (10/50, 20%) low-grade tumors were seen, which is similar to previous studies. Perhaps initial stages of canine lymphoma go unrecognized by most owners. Very few studies have focused on differentiation between T- and B-cell lymphomas in dogs because specific differential antibodies have only become available recently. The findings in this study show a good correlation between cytomorphology and immunophenotype. As in human medicine, the morphology of canine lymphomas may be suggestive of their phenotype. In a study, T-cell lymphomas were irregular nuclear outlines and abundant pale cytoplasm. These results corroborate recent findings in canines and humans. However, our results disagree with another study in which no correlation was found between morphology and immunophenotype. The use of nonspecific T-cell markers in the latter study might be responsible for the lack of correlation.

Past studies have found the proportion of T-cell lymphomas among canine lymphomas to range from 26% to 37.9%. In this study, the proportion of canine T-cell lymphomas was 36%. T-cell lymphomas in humans were 12% of cases reported by the NHL classification project. The data in this study indicate that most of the T-cell lymphomas belonged to the high-grade group (14/18, 78%) and only 4 (22%) to the low-grade group. These findings are similar to those of Teske et al., who observed that 80% of the lymphomas were high grade, but differ from those of Fournel-Fleury et al., who observed that 63% (15/24) of the lymphomas were low grade. The second most frequent subtype in this study was pleomorphic, mixed, small and large cell lymphoma, which constitutes a major category (5/18, 28%) within canine T-cell lymphomas. This is in agreement with other reports and with current human data indicating a prevalence of up to 58% T-cell type NHLs. The proportion of T-cell lymphoblastic lymphomas was higher (2/18, 11%) than that reported previously in some veterinary studies with or without determination of immunophenotype [(1/24, 4.2%) and (1/176, 0.6%)], lower than that reported in other veterinary studies [(49/285, 17.2%) and (11/46, 23.9%)], but close to the percentage of human lymphoblastic T-cell lymphomas (17%). In theory, lymphoblastic T-cell lymphomas are morphologically difficult to distinguish from their B-cell equivalents. But, in practice, the presumption of a T phenotype is high among lymphoblastic T-cell lymphomas. Indeed, Fournel-Fleury reported that among 140 cases of lymphomas all the lymphoblastic lymphomas had a T-phenotype. Similarly, more than 90% of the human lymphoblastic lymphomas with morphology very similar to their canine equivalents have T-phenotype. Unclassifiable high-grade plasmacytoid lymphomas are pleomorphic, with plasmacytoid features suggestive of B phenotype. However, the presence of irregular serrations in the apical part of the nuclei and the lack of the various cell components found in the pleomorphic lymphomas suggest a T-phenotype. Morphological findings and high Ki-67 index indicated that this group of lymphomas belonged to the high-grade category. Consequently, these lymphomas have been designated as unclassifiable high-grade plasmacytoid lymphomas.

In this study, 64% of lymphomas (32/50) were of B-cell origin, which is consistent with other studies. Similarly, Carter et al. reported in a study of 285 dogs with lymphoma that 70% of the tumors were of B-cell origin, 25% were of T-cell origin, and 5% expressed no distinct immunophenotype.
In this study, we identified low-grade lymphoma, which is similar to the one described by Magnol et al. as “lymphoma with macronucleolated medium sized cells (MMC).” This type has been described by Fournel-Fleury et al. who suggest that the cell arises from the marginal perifollicular zone. The MMC were classified as low-grade lymphomas on the basis of low mitotic activity and low expression of the Ki-67. However, one author has suggested that they are of a high grade of malignancy because of their clinically aggressive behavior and larger cell size.

Among the high-grade B phenotype lymphomas, 69% (18/26) were of the centroblastic type, similar to those described by other workers. In centroblastic lymphomas the percentage of centroblasts appears to be variable, which has led to their description as “polymorphic lymphoma with a centroblastic component” by Fournel-Fleury et al. In this study, a high percentage of large cells including immunoblasts characterized by a single nucleolus, or several nucleoli in a paracentral position, and extended hyperbasophilic cytoplasm resulted in a subclassification of “Centroblastic, predominantly large cell.” They accounted for 15% (4/26) of high-grade B-phenotype lymphomas, which is similar to the percentage described by Fournel-Fleury et al. Placing both subtypes of polymorphic predominantly large cell and predominantly small cell lymphomas in the same category would be justified by the fact that the small noncleaved, centroblastic, and immunoblastic cells may constitute successive evolutive stages. In this study, we identified 4 small lymphomas characterized by small noncleaved nuclei with plasmacytoid appearance, a high mitotic index, and B-phenotype. This tumor type has been reported previously only in dogs, and no human equivalents have been described. The proportion of lymphoblastic B-phenotype lymphomas seems to be higher (4/50, 8%) than that reported previously in most studies ([0/92], [4/176, 2.3%], and [3/61, 4.91%]) but similar to 1 previous report (49/285, 17.2%).

The average ages of dogs affected by T-cell (7.9 years) and B-cell (8.35 years) lymphomas was within the range reported previously. Canine T-cell lymphomas do not show any sex predilection. In contrast, males have a higher prevalence of B-cell lymphomas than females. The overrepresentation of Boxers observed in this study is in agreement with other veterinary data suggesting a genetic, familial predisposition to lymphoma in this breed.

No lymphomas tested in this study expressed a double (B and T) phenotype, which is in agreement with the results of other recent studies but in disagreement with results from earlier reports. Perhaps, the discrepancy can be explained by the lack of specificity of the pan-T markers used by the earlier authors.

This study described B- and T-cell lymphomas with the presence of a newly recognized type provisionally designated plasmacytoid T-cell lymphoma. At present, there is no such entity in the Revised European American Lymphoma (REAL), the World Health Organization (WHO), and updated Kiel classifications. More studies are needed to determine whether this is indeed a separate entity or merely a subtype of the unspecified peripheral T-cell lymphoma.

The combination of FNA cytology and FC in this study appears to be an accurate method for the diagnosis, immunophenotyping, and classification of canine lymphomas. There are few reports on the use of FNA for diagnosis, immunophenotyping, and classification of lymphomas in veterinary medicine. Immunophenotyping is important in the prognosis and therapy of canine lymphomas because the T-cell phenotype is associated with a high rate of relapse and significantly shorter survival times. In this study, the combination of cytological and phenotypic data with the use of updated Kiel classification proved to be useful for the classification of low-grade and high-grade lymphomas.

Sources and manufacturers

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b. Serotec, Oxford, UK.
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