Antigen-driven Clonal Proliferation, Somatic Hypermutation, and Selection of B Lymphocytes Infiltrating Human Ductal Breast Carcinomas

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ABSTRACT

Infiltration of B lymphocytes into the tumor tissue of breast cancer patients is a common occurrence, but the role of these cells in the immune response to the tumor is unknown. Heavy B-cell infiltration in medullary breast carcinoma is well documented and associated with a more favorable prognosis, implying a positive role for the humoral immune response in elimination of tumor cells. Variable B-cell infiltration has also been detected in infiltrating ductal carcinomas of the breast, but little is known about the immunoglobulin gene repertoire of these tumor-infiltrating B lymphocytes and whether they are actively responding to a local stimulus or merely passive bystanders. We have therefore investigated the repertoire of B cells infiltrating four invasive ductal carcinomas. A group of 233 rearranged Ig VH genes was amplified, cloned, and sequenced from microdissected foci of infiltrating B cells. B cells within individual foci were polyclonal, and most were highly mutated. Several foci expressed dominant sets of V genes derived from B-cell clones. Some of these were found in more than one lymphoid cluster, indicating that B cells had migrated into the surrounding tissue and seeded new clusters. Analysis of the pattern of mutations in clonally related sets of Ig V genes expressed by tumor-infiltrating B cells shows that these cells are undergoing antigen-driven proliferation, somatic hypermutation, and affinity maturation in situ.

INTRODUCTION

Breast carcinoma cells are often closely associated with TILs in the primary tumor and metastatic tumors in the axillary lymph nodes (1, 2). Although the exact role of lymphocyte infiltration of primary carcinomas has not been elucidated, it may reflect a host response to the tumor (2). The inverse correlation between the extent of B-cell infiltration and tumor size and grade in medullary breast carcinoma (3, 4) may be indicative of a favorable and partially successful host response against the tumor. Invasive ductal carcinomas are the most common type of breast cancer, accounting for 80% of cases (5), but unlike medullary breast carcinoma, they have more variable B-lymphocyte infiltration. It has been suggested that these TILs might be abnormal or defective because they migrate into the tumor and yet, in most cases, fail to contain its growth (6). Interestingly, TILs are significant predictors of recurrence-free survival in women with early onset breast cancers but not in older women (7).

Studies show that the lymphocytic infiltrates in ductal breast cancer consist largely of T cells, with variable numbers of macrophages, natural killer cells, and B cells (8, 9). Presently, there is disagreement on the T-lymphocyte subpopulations. Although some investigators report CD4+ T cells as predominant, especially in large tumors (6, 9), others show a higher proportion of CD8+ T lymphocytes in breast cancer and melanoma (8, 10). Because of the abundance of T lymphocytes in tumor tissue, most in-depth studies on TILs have concentrated on T and natural killer cells (6, 11–13).

In contrast, tumor-infiltrating B cells are poorly characterized in their specificity and Ig variable region repertoire. Very little is known about the significance of antibody responses to a tumor (14), despite the presence of TIL B cells in $\geq 20\%$ of breast cancers (6).

B cells generate Ig (antibody) diversity by several mechanisms. In the case of heavy chains, two recombination events bring together different VH, DH, and JH exons, and short additional sequences inserted between VH and DH and also between DH and JH generate further diversity. All of these occurs in the bone marrow during the formation of a naïve B cell, before exposure to antigen, and comparable events occur in the light chain genes. Subsequent encounter with antigen in the appropriate cellular environment drives B-cell clonal proliferation and somatic hypermutation in germinal centers of secondary lymphoid organs, which generates further antibody diversity. Cells which express mutated antigen receptors with higher affinity for the stimulating antigen are selected by a second encounter with antigen on the surface of specialized antigen-presenting cells within germinal centers (FDCs). This leads to the generation of successive cycles of B cells expressing antigen receptors with higher affinity for antigen than the one before it. At the same time, selected B cells differentiate into memory B cells and antibody-secreting plasma cells.

The aim of this work was to use our knowledge of these molecular events to investigate the role of the tumor-infiltrating B lymphocytes present in ductal carcinomas, to explore the nature of the B-cell response to breast cancer. Our data demonstrate that B cells present in clusters within or in close proximity to tumor tissue do not migrate independently into the tissue after stimulation in peripheral lymphoid organs but are stimulated locally by antigen.

MATERIALS AND METHODS

Tumor Samples. Four patients undergoing therapeutic excision of breast carcinomas were included in this study after informed consent. Ethical approval for the study was granted by the North Glasgow Hospitals University National Health Service Trust Research Ethics Committee. No patient had received previous chemotherapy, radiotherapy, or endocrine manipulation. Carcinoma samples were embedded in Tissue-Tek OCT Compound (Sakura, Zoeterwoude, the Netherlands) and snap frozen in liquid nitrogen immediately after surgery. Serial frozen sections (8 μm) were cut with a cryostat and mounted on coated slides (Menzel Glaser, Germany). Sections were air dried, fixed in acetone, and stored at −80°C with a desiccant.

Immunohistochemistry. Sections were stained with mouse monoclonal antibodies specific for B cells [anti-CD20 (1:50); DAKO, Carpinteria, CA], T cells [anti-CD3 (1:100); DAKO], FDCs [anti-FDC (1:100); DAKO], and plasma cells (Wue-1; A. Greiner, University of Würzburg), followed by rabbit antimiouse Ig (1:20; DAKO) and alkaline phosphatase antialkaline phosphatase complex (1:50, DAKO). Immune complexes containing alkaline phosphatase antialkaline phosphatase were detected by incubation with new fuchsin substrate, and the sections were counterstained with Mayer’s hematoxylin (Sigma; Poole, United Kingdom).

Microdissection of Tumor-infiltrating B Cells and DNA Extraction. B-cell clusters were excised from frozen sections (8 μm) under sterile distilled water using sterile skin-prick lancets controlled by micro-manipulators (Narishige, Tokyo, Japan) under a Nikon Diaphot (Melville, NY) inverted microscope. The excised tissue was digested in 30 μl of protease K (0.7 mg/ml;
Boehringer Mannheim, Mannheim, Germany) at 50°C for 1 h, and the enzyme was inactivated at 95°C for 10 min. To avoid contamination, a fresh lancet was used for each B-cell cluster and discarded if it came into contact with any site other than the target B-cell cluster. In addition, all procedures before PCR were performed in a clean laboratory separate to that where postamplification steps were carried out.

Amplification and Cloning of Rearranged Heavy Chain V Genes. Rearranged \( V_H \) genes were amplified by nested PCR. The primers were designed to amplify all known functional, rearranged human Ig \( V_H \) genes and have been published previously (15). Amplification and cloning of rearranged heavy chain \( V \) genes were carried out as described (16) except that the annealing temperature was increased to 62°C in the primary PCR.

Sequencing and Analysis of Rearranged Genes. Plasmid DNA containing gene inserts was prepared using QIAprep spin mini-prep kits (Qiagen, Chatsworth, CA), precipitated, washed three times, and resuspended in sterile 1x TE buffer (pH 7.5). \( V_H \) genes were sequenced by ABI automated cycle sequencing (Applied Biosystems, Foster City, CA) in both directions. Sequences were compared with the human VBASE directory of Ig genes (17) using DNAplot (W. Müller, Institut für Genetik, Köln, Germany) to identify the best matching germ-line gene segments. The nomenclature of \( V, D, \) and \( J \) gene segments adopted here and the definitions of CDRs have been described previously (18). \( V \)-gene sequences belonging to a single B-cell clone were identified on the basis of identical \( V_H, D_H, \) and \( J_H \) gene usage and \( V-D, D-J \) junction sequences. The pattern of mutations for each sequence was compared with other sequences from the same patient and germ-line genes to identify hybrid sequences derived from recombinant \( V_H \) gene segments. Genealogical trees showing the relationships between B-cell clones were constructed by analysis of shared and unshared mutations.

Statistical Analysis. The distribution of \( V_H \) and \( J_H \) gene family usage was assessed using \( \chi^2 \) analysis.

RESULTS

Characterization of Infiltrating Lymphoid Cells in Ductal Breast Carcinoma Tissue. Infiltrating B cells were present in all four carcinomas studied (Fig. 1, A–D), and all of the cell types found in the germinal centers of normal lymphoid tissue were also detected.

![Image](image_url)
in breast carcinoma lymphoid infiltrates. B and T cells were frequently present in close proximity in the same areas, sometimes in discrete clusters. In three carcinomas, greater numbers of T than B lymphocytes were present. The exception was the carcinoma of patient D, in which equal numbers of B and T cells were present. FDCs and plasma cells were also present in B-cell clusters in all carcinomas. The density of FDCs correlated with that of B cells, and FDCs were confined to the areas infiltrated by B cells. In contrast, plasma cells were found both within and dispersed around areas of B-cell infiltration. The number of B cells dissected from the clusters was estimated by counting the cells across the axes in the anti-CD20-stained sections. Assuming that the shape of the clusters is approximately circular, the small clusters from patients A, B, and C contained \( \approx 5000 \) B cells, whereas the larger clusters from patient D contained \( \approx 6 \times 10^4 \) cells.

**The Repertoire of Ig Heavy Chain V Genes Expressed by Tumor-infiltrating B Cells.** Analysis of V<sub>H</sub> gene usage indicated that the V<sub>H</sub>3 gene family was used most often (Fig. 2). V<sub>H</sub>3-23/DP 47 and V<sub>H</sub>5-51/DP73 clearly predominated, being used by 30 (30.9%) of the functional rearrangements in the tumor tissue. Use of V<sub>H</sub> gene families differed significantly in breast tumor from normal subject peripheral blood lymphocyte (19) and theoretical expected frequencies (\( P = 0.000015 \) and <0.0001, respectively).

I<sub>H</sub> gene usage showed a clear bias toward I<sub>H</sub>2 (and, to a much lesser extent, I<sub>H</sub>6), at the expense of I<sub>H</sub>1 and I<sub>H</sub>2 (Fig. 2; \( P < 0.001 \)). All seven D<sub>H</sub> gene families were found in the V genes from the four patients, with D<sub>H</sub>3 and D<sub>H</sub>6 being most frequently used (data not shown).

As described, a B-cell clone is defined by the set of related V<sub>H</sub> gene sequences derived from the same germ line, with identical V-D-J rearrangements and identical junctions, but differing by several nucleotides over the V<sub>H</sub> region, resulting from somatic mutations (20). The number of rearranged V<sub>H</sub> genes and clonally related sets is summarized in Table 1.

**Functional V-Gene Rearrangements.** A group of 201 V<sub>H</sub> gene sequences derived from 97 independent functional V-gene rearrangements was isolated from eight B-cell clusters in tumor tissue from the four patients. TIL B-cell clusters from each patient expressed diverse V-gene rearrangements using a variety of V<sub>H</sub>, D<sub>H</sub>, and J<sub>H</sub> gene segments. Three pairs of nonclonally related sequences using the same V<sub>H</sub>, D<sub>H</sub>, and J<sub>H</sub> gene segments but with different junctional sequences were present in patient D (Table 2). In addition, two sequences (one each from patients A and B) also used the same V<sub>H</sub>, D<sub>H</sub>, and J<sub>H</sub> gene segments but had different junctions, suggesting a strong selection pressure for these combinations of gene segments. In all of the patients, the number of somatic mutations within each V gene varied widely, with the majority of sequences having acquired between 11 and 20 postrearrangement mutations, (Fig. 3). The B-cell clone identified in patient B (Clone B) was represented in sequences from two different B-cell clusters (Fig. 4, sequences G and N originated from a different cluster from the other sequences).

In addition to the 10 functional B-cell clones identified in the four patients, five other sets of related sequences consisting of at least three members were identified. These five sets averaged one nucleotide difference per V<sub>H</sub> gene and may therefore also have been derived from clones of B cells. They were not included in the analysis, although they were significantly above the PCR error rate of one base per four VH genes (16). This cautious approach may underestimate the true diversity, because it probably ignores early B-cell clones.

**Nonfunctional Rearrangements.** Among the 233 sequences examined, 32 (14%) were nonfunctional, mostly because of out-of-frame V-D-J rearrangements. A nonfunctional set of clonally related sequences was detected in patient C. Presumably, a clone of B cells that had undergone a secondary functional rearrangement, producing a viable B-cell receptor, carried these nonfunctional genes.

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**Table 1**  
Summary of V<sub>H</sub> genes isolated from B-cell clusters

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of B-cell clusters dissected</th>
<th>No. of unrelated V genes</th>
<th>No. of clonally related sets*</th>
<th>Hybrid V-D-J rearrangements</th>
<th>Total no. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>7</td>
<td>2 (26)</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>7</td>
<td>1 (14)</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>8</td>
<td>1 (6)</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>65</td>
<td>6 (32)</td>
<td>17</td>
<td>88</td>
</tr>
</tbody>
</table>

* The number of all sequences corresponding to each group of genes is shown in parenthesis.

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**Table 2**  
Assignment of best matching germ-line variable region genes to the clonally related sets isolated from the four patients and analysis of the V-gene somatic mutation rate (clones A1 and A2 were derived from patient A, clone B from patient B, clones C1 and C2 from patient C, and clones D1-6 from patient D)

<table>
<thead>
<tr>
<th>Clonally related sets</th>
<th>Germ-line gene</th>
<th>Range of mutations/sequence</th>
<th>CDR mutations</th>
<th>FR mutations</th>
<th>CDR R:S ratio</th>
<th>FR R:S ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-23</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;3-22</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;6-03</td>
<td>0–10</td>
<td>11 (2)</td>
<td>6 (0)</td>
</tr>
<tr>
<td>A-2</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-7</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;3-16</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;1-02</td>
<td>32–33</td>
<td>20 (5)</td>
<td>9 (6)</td>
</tr>
<tr>
<td>B</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-7</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;3-16</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;3-02</td>
<td>17–27</td>
<td>15 (4)</td>
<td>13 (6)</td>
</tr>
<tr>
<td>C-1</td>
<td>VH3-23</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;3-10</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;6-03</td>
<td>12 (1)</td>
<td>25 (6)</td>
<td>12</td>
</tr>
<tr>
<td>C-2(NF)*</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-1</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;4-02</td>
<td>7–9</td>
<td>9 (1)</td>
<td>3 (2)</td>
<td>9</td>
</tr>
<tr>
<td>D-1</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;8-09</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;3-9</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;6-02</td>
<td>6–9</td>
<td>13 (3)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>D-2</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;5-11</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;6-19</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;4-02</td>
<td>9–24</td>
<td>17 (3)</td>
<td>10 (2)</td>
</tr>
<tr>
<td>D-3</td>
<td>VH5-51</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;6-02</td>
<td>23–37</td>
<td>18 (2)</td>
<td>13 (6)</td>
<td>9</td>
</tr>
<tr>
<td>D-4</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-33</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;6-19</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;4-02</td>
<td>14–24</td>
<td>9 (1)</td>
<td>15 (6)</td>
</tr>
<tr>
<td>D-5</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-23</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;7-27</td>
<td>J&lt;sub&gt;J&lt;/sub&gt;2-01</td>
<td>18–19</td>
<td>9 (4)</td>
<td>4 (5)</td>
</tr>
</tbody>
</table>

* Nonfunctional. Replacement and silent mutations were calculated for both FR and CDR. The silent mutations are shown in parenthesis. Only clone A1 included unmutated sequences (5).
B-cell Clones Are Proliferating within the Tumor Tissue. Related sequences with identical V-D-J rearrangements and identical junctions but differing by several nucleotides over the V_H region were identified in all four patients. Ten sets of related sequences exhibiting significant mutation rates were identified as members of B-cell clones (Table 2). These results demonstrate that B-cell clonal proliferation is occurring in clusters of B cells within the tumor tissue in ductal breast carcinoma. Genealogical trees illustrate the evolution of sequences in clonally related sets from the germ-line sequence (Fig. 4). The four trees illustrated were chosen to represent clones isolated from all four patients and illustrate the different evolutionary pathways identified. Clone A1 contained unmutated V genes, as well as mutated variants,

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**Fig. 4.** B-cell clones from all four patients show clonal proliferation and somatic hypermutation. Clones are shown as proliferating from the best matching germ-line VH gene segment. Each circle symbolizes a B cell, and letters within depict individual sequences. Deduced intermediates are shown as dotted circles. The numbers alongside the arrows represent mutations between the different sequences. In clone B, sequences G and N were from a different cluster from the rest of the sequences.
demonstrating that the clone was recently derived from a naïve B cell that had migrated into the tumor tissue. Although the earliest derived precursors from the other nine clones contained between 4 and 28 mutations, it is possible that unidentified, unmutated precursors were also present in the breast tissue but were not identified.

**Somatic Hypermutation in the V Genes of Tumor-infiltrating B Cells.** Most of the functionally rearranged sequences from the four patients were highly mutated, with between 11 and 20 mutations (Fig. 3). Clonally related sets of V genes were often highly mutated. The numbers of silent and replacement mutations in the CDRs and FRs were calculated in the identified B-cell clones to determine whether or not selection of particular mutations by antigen had occurred, as takes place in germinal centers. The ratio of replacement to silent mutations (R:S ratio) in the CDRs varied widely, with no correlation with the number of mutations. Generally, high R:S ratios in the CDR (all sets except D-6) and low R:S ratios in the FR (all sets except D-3) indicate antigen-driven clonal selection (Table 2).

**B-cell Clusters Are Composed of Several B-cell Clones.** B-cell clones A1 and A2 originated from the same cluster, with neither clone predominating. Similarly, clones D2 and D5 were also from the same cluster, with neither clone predominating. On the other hand, clone D4 with 18 sequences was much larger than clone D6 with four members.

**DISCUSSION**

We investigated the Ig gene repertoire of tumor-infiltrating B cells present in lymphoid cell clusters within, or in close proximity to, the tumor tissue from four ductal breast carcinoma patients. The clusters of B and T cells did not have the histological appearance of typical follicular germinal centers, as found in the lymph nodes and spleen, in that they did not have a well-defined mantle zone of small B cells around a germinal center comprising dark and light zones. Nevertheless, all of the components required for antigen-driven B-cell proliferation and maturation in a germinal center, i.e., T and B cells and FDCs, were present in the cell clusters. The presence of FDCs is particularly significant, because they are normally localized to germinal centers in peripheral lymphoid tissues and play a crucial role in affinity maturation by antigen selection of B cells with high affinity mutated antigen receptors (21, 22). Similar clusters of B and T cells and FDCs have been observed in the target tissues of autoimmune diseases, such as Sjögren’s syndrome and rheumatoid arthritis (23, 24), and in the thymus of patients with myasthenia gravis (16).

Ig V genes isolated from members of the same B-cell clone were identified by their use of the same germ-line VH, D, and J exons and identical sequences at the VH-D and D-JH junctions, indicating that they were derived from the same progenitor B cell. The presence of shared and unshared somatic mutations within the V genes allows the construction of genealogical trees of clonally related B cells. Analysis of the sequences clearly demonstrates oligoclonal expansion of B cells undergoing somatic hypermutation within the clusters of lymphocytes in the tumors. This resembles the B-cell response in mature germinal centers of secondary lymphoid organs, which are usually derived from only one to three founder B cells (25, 26). The founder cells of most of these clones were highly mutated, only clone A1 being founded by a B cell expressing a germ-line VH gene. It is likely, therefore, that many tumor-infiltrating B-cell clones arise from memory B cells that migrate into the tumor after clonal expansion and differentiation in the peripheral lymphoid organs. Unusually, one clonally related set of V genes (clone B) contained sequences isolated from two different clusters in close proximity to each other, suggesting that cells are able to migrate out of one cluster into the surrounding tissue and seed new clusters. Although we cannot exclude the possibility that they seeded the two clusters independently from a draining lymph node, we did not find clonally related V genes in any of the spatially separated clusters. This has also been observed in the germinal center response to xenoantigens in the spleen (26) but not in ectopic germinal centers in the autoimmune diseases Sjögren’s syndrome (23) and myasthenia gravis (16).

It is well established that B cells undergoing a germinal center response, i.e., clonal proliferation accompanied by somatic hypermutation and affinity selection, are driven by antigen, which is required as both the initial trigger and at the later stage of affinity selection (25, 27). Our observation of clonally related sets of mutated V genes expressed by B cells within the cell clusters shows that TIL-B cells are undergoing a similar process of antigen-driven clonal proliferation and affinity maturation in situ.

Analysis of VH gene usage revealed biased use of VH5–51 and VH3–23, of which only VH3–23 is ordinarily over-represented in the peripheral blood B-cell repertoire of normal individuals (19, 28). It is unlikely that this bias was introduced by PCR, because the mixtures of primers had been optimized to maximize the diversity of PCR products, and the same primers have been extensively used to amplify V genes from other tissues without showing the same pattern of repertoire expression (15, 16). We postulate that the presence of dominant VH transcripts may itself be a consequence of antigen-driven selection and oligoclonal proliferation. This is supported by the results of our analysis of replacement (R) and silent (S) mutations in the CDR and FR regions of the V genes.

Although some regions of the VH gene, notably CDR1 and CDR2, are more susceptible to mutations leading to amino acid replacements (29), it is generally thought that the distribution and frequency of mutations within the V gene can be used to distinguish those antibody sequences driven by antigen selection of a B-cell clone from those containing random mutations. With one exception (clone D3), our results showed a higher proportion of replacement mutations in the CDRs than in the FRs (Table 2), a typical feature of affinity matured antibodies (29). A high replacement:silent ratio in the CDRs is believed to result from selection of mutations providing the best “fit” for the antigen in the antigen-binding site, whereas the lower ratio in the FRs preserves the domain structure supporting the antigen-binding site. Replacement mutations in the CDRs which improve antibody affinity are therefore selected for, whereas replacement mutations in the FRs, which disrupt domain structure, are selected against.

In conclusion, our study suggests that B cells infiltrating ductal breast carcinoma tissue are undergoing antigen-driven clonal proliferation and affinity selection in situ. Our conclusions are further supported by a recent study (30) showing evidence for oligoclonal expansion of B cells infiltrating ductal carcinoma, based on V-gene sequences from whole tumor tissue. Additional studies are needed to identify the antigens driving this response and will provide additional information on the nature of the B-cell response in ductal breast carcinoma.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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