Rationale for Development of a Synthetic Vaccine against Plasmodium falciparum Malaria
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centrations of these organophosphatase pollutants are high enough to exert selective pressure (20). In some cases, microorganisms may have the enzymatic capability to degrade the organophosphatase but the organophosphatase may not be present in sufficient concentrations to induce the enzymes required for degradation. In either case, pollutants present in low concentrations will not be degraded. In P. chrysosporium, the degradation of organohalides and of lignin is initiated by nitrogen starvation rather than by the presence of substrate. Thus large concentrations of organohalides or other recalcitrant pollutants need not be present to induce the enzymes required to initiate biodegradation.

Phanerochaete chrysosporium and related fungi (in the class Basidiomycetes where there are between 1600 and 1700 species of wood-rotting fungi) are responsible for recycling carbon bound in lignin (21). Their action might be important in the biodegradation of persistent man-made organic compounds in the environment. Numerous strategies have been used in the aerobic treatment of contaminated waste effluents, sludges, sediments, and landfills. Among these are activated sludge processes, aerated lagoons, aerobic digestion, trickling filters, rotary biological contactors, and aerobic composts (22). The effectiveness of these systems is ultimately dependent upon the microorganisms present in the system. Thus it is critical that the most appropriate organisms (those with a demonstrated inherent ability to degrade a wide range of environmental pollutants) be selected. We propose that biotreatment systems inoculated with P. chrysosporium and fortified with a suitable carbohydrate source, under nitrogen-limiting conditions, may provide an effective and economical means for the biological detoxification and disposal of hazardous chemical wastes.

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Rationale for Development of a Synthetic Vaccine Against Plasmodium falciparum Malaria

Abstract. Protective immunity against malaria can be obtained by vaccination with irradiated sporozoites. The protective antigens known as circumsporozoite (CS) proteins, are polypeptides that cover the surface membrane of the parasite. The CS proteins contain species-specific immunodominant epitopes formed by tandem repeated sequences of amino acids. Here it is shown that the dominant epitope of Plasmodium falciparum is contained in the synthetic dodecapeptide Asn-Ala-Asp-Pro-Asn-Ala-Asp-Pro-Asn-Ala-Asp (or (NANP)). Monoclonal antibodies and most or all polyclonal human antibodies to the sporozoites react with (NANP), and polyclonal antibodies raised against the synthetic peptide (NANP) react with the surface of the parasite and neutralize its infectivity. Since (NANP) repeats are present in CS proteins of P. falciparum from many parts of the world, this epitope is a logical target for vaccine development.

The development of vaccines against malaria is complicated by the fact that the protective antigens are specific for each of the main developmental stages of the parasite, Plasmodium falciparum, in the human host. These stages include the sporozoites, which are injected by mosquito bite; the blood stages, which develop in red cells and cause the clinical disease; and the gametocytes, which are infectious for Anopheles mosquitoes. An effective vaccine against the sporozoites would be most advantageous because it would block infection in the human and

5. T. K. Kirk, in Microbial Degradation of Organ-
prevent transmission of the disease. Even if sterile immunity is not achieved in all individuals, the decrease of sporozoite load may diminish the severity of the disease and mortality (7).

Protective immunity against sporozoites has been achieved by inoculation of relatively small numbers of x-irradiated parasites into rodents, monkeys, and humans (2). The immunity is usually species-specific. Incubation of sporozoites with the sera of vaccinated and protected animals results in the formation of a tail-like precipitate (circumsporozoite or CSP reaction) and in the abolishment of parasite infectivity. The target antigens of these reactions have been identified by monoclonal antibodies. They consist of single polypeptides (circumsporozoite or CS proteins) that cover the entire surface membrane of the parasite and are shed when cross-linked by antibodies. All CS proteins contain a species-specific immunodominant domain displaying repeated epitopes (3).

The genes encoding the CS proteins of *Plasmodium knowlesi* (H strain) (4, 5), *Plasmodium cynomolgi* (Gombak) (6), and the human malaria parasite *P. falciparum* (7, 8) have been cloned and the structures of the polypeptides elucidated. The immunodominant epitopes are located within a large domain of the CS molecule formed by tandem repeated sequences of amino acids, NNP in *P. falciparum*, GQPQAGGQGAGA in *P. knowlesi*, and GAAAAGGGGN in *P. cynomolgi*. Monoclonal antibodies (or their Fab fragments) to this domain neutralize the in vitro infectivity of sporozoites by preventing their attachment to hepatocytes (9). The characterization of the corresponding epitopes, which are likely to be involved in the initial interaction between the parasite and target cell, is therefore of potential importance for vaccine development.

To determine the structure of the immunodominant epitope of *P. falciparum*, we synthesized a series of synthetic peptides, (NANP)₂, (NANP)₃, (NANP)₄ (10), and used them to inhibit the binding of monoclonal antibodies to extracts of sporozoites. The results (Fig. 1) showed that (NANP)₃ and (NANP)₄ strongly inhibited the binding of the antibodies to the antigen with almost equal efficiency on a molar basis. In contrast, (NANP)₂ was a poor inhibitor. Similar findings have been reported by others (7) using recombinant products expressed in bacteria as target antigens.

In view of these results, we used (NANP)₃ as the antigen for an immunoradiometric assay (IRMA) to detect antibodies to sporozoites in the sera of humans living in an endemic area. The Gambia, West Africa. Fifty-eight blood samples were collected randomly from children and adults during November and December 1982 at the Clinic of the Medical Research Council in Fajara. In agreement with previous epidemiological studies showing that the immune re-

![Image](https://example.com/image.png)

**Fig. 1. Inhibition of binding of four monoclonal antibodies against *P. falciparum* sporozoites by synthetic peptides.** The antibodies, 2A10, 2C11, 2E7, and 3D6, at a concentration of 50 ng/ml were incubated with increasing concentrations of peptide. After 1 hour at room temperature, 30 μl of the mixtures were placed in duplicate wells of *P. falciparum* sporozoite coated plates prepared as described (3). After incubation for 1 hour, the wells were extensively washed with phosphate-buffered saline (PBS) containing 1 percent of bovine serum albumin (BSA) and 0.05 percent Tween-20. Then 30 μl of 125I-labeled affinity purified goat antibody to mouse immunoglobulin (Kirkwood and Perry Laboratories) were placed in each well, incubated for 1 hour, washed three times with PBS-BSA, dried, and counted in a gamma counter. Symbols: ●, (NANP)₂; ○, (NANP)₃; □, (NANP)₄; △, controls with no peptides.

<table>
<thead>
<tr>
<th>Serum</th>
<th>IRMA with (NANP)₃ as antigen (cpm)*</th>
<th>IFA with glutaraldehyde-fixed sporozoites as antigen</th>
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<tr>
<td></td>
<td></td>
<td>Serum titer</td>
</tr>
<tr>
<td>G.Z.</td>
<td>9201</td>
<td>4096</td>
</tr>
<tr>
<td>IDA</td>
<td>4851</td>
<td>1280</td>
</tr>
<tr>
<td>8017</td>
<td>3539</td>
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</tr>
<tr>
<td>7930</td>
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<td>640</td>
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<td>320</td>
</tr>
<tr>
<td>P-2</td>
<td>2473</td>
<td>320</td>
</tr>
<tr>
<td>P-5</td>
<td>2024</td>
<td>320</td>
</tr>
<tr>
<td>8012</td>
<td>1765</td>
<td>640</td>
</tr>
<tr>
<td>Normal</td>
<td>163</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>&lt;10</td>
</tr>
<tr>
<td>8074</td>
<td>133</td>
<td>20</td>
</tr>
<tr>
<td>7878</td>
<td>96</td>
<td>&lt;10</td>
</tr>
<tr>
<td>P-12</td>
<td>75</td>
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<td>8312</td>
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<td>&lt;10</td>
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<tr>
<td>8286</td>
<td>91</td>
<td>20</td>
</tr>
<tr>
<td>7907</td>
<td>103</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

*The IRMA antigen was (NANP)₃, immobilized by means of glutaraldehyde in plastic wells coated with BSA. To saturate the remaining glutaraldehyde reactive groups, the wells were treated with 0.5 M ethanolamine. Serum samples were ten times diluted in a mixture of BSA and PBS containing 0.5 M ethanolamine and 0.05 percent Tween-20, and 20 μl were placed in each well. After 1 hour, the wells were washed and incubated with 125I-labeled affinity-purified goat antibodies to human immunoglobulins to reveal the presence of bound antibodies. Each serum was simultaneously tested in duplicate peptide-coated wells and in control wells prepared as described above but omitting the peptide. The mean radioactivity in control wells, which varied from 200 to 500 count/min, was subtracted from the radioactivity in the corresponding peptide-coated wells and the mean of the difference defined as cpm. When the results of IFA and IRMA were compared by a nonparametric (Wilcoxon rank correlation), the r was 0.87 (P < 0.001). †Serum samples were incubated with 50 μg (NANP)₃ per milliliter for 2 hours at room temperature before performing the IFA, ND, not done.

*From G.Z., a human volunteer vaccinated with irradiated *P. falciparum* sporozoites and protected against malaria infection.
sponse of humans to sporozoites is age-
dependent (11, 12), the percentage of positive sera detected by the IRMA in-
creased with age, ranging from 21 per-
cent in children 1 year old to 95 per-
cent in adults older than 34 years. Very high levels of antibodies to
(NANP)_2 were also found (Table 1) in
the serum of a human volunteer (G.Z.)
vaccinated with x-irradiated P. falciparum sporozoites and protected against
malaria infection (13).

Next we used an indirect immunofluo-
rescence assay (IFA) to detect antibod-
ies to sporozoites in randomly selected
IRMA-negative and IRMA-positive sera
from individuals older than 20 years. The
results (Table 1) showed a significant
Spearman rank correlation coefficient
between the IRMA and IFA titers (r_s = 0.87, P < 0.001). Among nine
IRMA negative sera examined, seven
were also negative by IFA and two had
very low IFA titers. Moreover, when the
positive sera from the endemic areas and
from the vaccinated human volunteer were
preincubated with soluble (NANP)_2 peptide, the IFA was greatly
reduced or abolished. More strikingly, in
these human sera most or all of the antibodies were directed to (NANP)_2
(14). In further experiments the human
antibodies failed to react with (NANP)_2
and the sensitivity of assay did not im-
prove by substituting (NANP)_4 for
(NANP)_2.

In view of these results, we immu-
nized several groups of rabbits with con-
jugates prepared by coupling (NANP)_2
to tetanus toxoid with glutaraldehyde.
We assayed the sera obtained 4 weeks
after immunization by an IRMA, using
(NANP)_2 immobilized on the bottom
of plastic wells as antigen. All samples from
immunized animals were positive, while
the reactivity of preimmune sera was
negligible. The positive reactions were
inhibited by preincubating the sera with
(NANP)_2 (25 μg/ml). The results with a
single batch of conjugate are summa-
rized in Fig. 2. Identical results were
obtained with two other preparations of
antigen (6). The three sera injected with
1 mg of antigen emulsified in incom-
plete Freund’s adjuvant, the titers (see
Fig. 2) varied between 1,000 and 10,000.
The results were similar when the same
dose of antigen was administered in com-
plete Freund’s adjuvant to three rabbits.
With tenfold less antigen (0.1 mg) emul-
sified in incomplete adjuvant, the titers
were lower, between 320 and 80. Anoth-
er group of three rabbits received, 2
weeks apart, two injections of 1 mg of
the conjugate without adjuvant. Their
serum titers 4 weeks after the first injec-
tion were between 80 and 10. In animals
receiving adjuvant the titer of antibodies
to (NANP)_2 did not decrease for at least
3 months after immunization.

The antibodies to (NANP)_2 reacted in
a Western blot with the P. falciparum CS

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Fig. 2 (left). Immunoassays on sera from rabbits injected with (NANP)_2-tetanus toxoid. Tetanus toxoid (from the Pasteur Institute) was diazylzed
exclusively against distilled water and lyophilized. The conjugate was obtained by incubation of 10 ml each of tetanus toxoid (1 mg/ml) and
(NANP)_2 (1 mg/ml) with 0.02 percent glutaraldehyde. After incubation for 6 hours at room temperature, the mixture was diazylzed for 72 hours
against distilled water, and lyophilized; 16 mg of the conjugate were recovered. Rabbits were injected in one hind foot pad and the opposite thigh
intramuscularly twice, 2 weeks apart, with 1 mg of conjugate (A and C). Blood was withdrawn 4 weeks after initial immunization. The IRMA was
performed as described in the footnote of Table 1, except that the plates were coated only with (NANP)_2. The results for each serum sample are
plotted. Titers as referred to in the text are the reciprocal of the serum dilution giving 10^6 counts per minute in the IRMA. This level of reactivity
fell within the linear portions of the titration curve which had almost identical slopes. The IFA titers with glutaraldehyde-fixed sporozoites used
as antigen (11) are given on the right side of the chart. Inset: Western blots of P. falciparum sporozoite extracts (7G8 Brazilian strain) revealed by
a rabbit antiserum to P. falciparum sporozoites (lane 1), against (NANP)_2-tetanus toxoid (lane 2), and normal rabbit serum control (lane 3). The
sporozoites were extracted in a buffer containing 2 percent sodium dodecylsulfate (SDS), 6M urea, and 10 percent glycerol for 3 minutes at 80°C
and then subjected to polyacrylamide gel electrophoresis (SDS-PAGE), with 5 percent and 10 percent stacking and separating gels. After the run,
the proteins were transferred to nitrocellulose paper. The paper strips were saturated with 5 percent BSA, and incubated with 1/20 dilutions of
rabbit antiserum. The bands indicated by arrows correspond to the precursor (67,000 daltons) and membrane forms (58,000 daltons) of the CS
protein as previously determined by using monoclonal antibodies. Some additional antigens revealed by the antisera against P. falciparum
sporozoites (lane 1) probably originate from contaminating material in the salivary glands of the infected mosquitoes. Fig. 3 (right). Inhibition
of binding of polyclonal antibodies to (NANP)_2, by P. falciparum sporozoite extracts. Serum from a rabbit immunized with (NANP)_2-tetanus
toxoid incorporated in incomplete Freund’s adjuvant. Samples of serum diluted 1/1000 in PBS-BSA were incubated with increasing amounts of
extracts of sporozoites of P. falciparum (7G8 Brazilian strain). The P. berghei sporozoites were from the salivary glands of Anopheles stephensi
mosquitoes. The sporozoites were counted, pelleted, resuspended in PBS containing 0.5 percent NP-40 for 1 hour at room temperature. The
extracts were centrifuged at 10,000g to remove insoluble materials. After 1 hour at room temperature, 30 μl of the mixtures were placed in
peptide-coated wells (see footnote to Table 1). After incubation for 1 hour, the wells were washed with PBS-BSA containing 0.5 percent Tween-
20 and incubated with 30 μl of 125I-labeled affinity purified goat antiserum to rabbit immunoglobulin. After 1 hour the wells were washed with
PBS-BSA and Tween-20, dried, and counted in a gamma counter.

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protein and its precursors, and with the surface of glutaraldehyde-fixed sporozoites of *P. falciparum*, as determined by IFA (Fig. 2). The sera with highest IFA and IRMA titers gave strong circumsporozoite reactions (titers of 1/50 to 1/100) when incubated with viable parasites.

A highly significant rank correlation was found between the IRMA and IFA titers ($r = 0.94; P < 0.001$), suggesting that most antibodies to (NANP)$_5$ recognized the CS protein. We therefore performed an IRMA on the antiserum after it had been incubated with increasing amounts of *P. falciparum* sporozoite extract (Fig. 3). About 70 percent of the reactivity of the antibody to (NANP)$_5$ was inhibited by the extract. Extracts of sporozoites of the rodent parasite *P. berghei* had no effect. In the converse experiment, we assayed the peptide for its inhibitory effect on the IFA titer of a rabbit antiserum to *P. falciparum* sporozoites. This antiserum was obtained by injecting a rabbit four times, at monthly intervals, with $10^9$ to $10^8$ sporozoites recovered from *Anopheles freeborni* mosquitoes fed on cultured blood forms of *P. falciparum* (15). Its IFA titer was $10^9$ but it dropped to below $10^3$ after preincubation with (NANP)$_5$ (50 µg/ml).

Immunoglobulin from the serum of a rabbit immunized with the (NANP)$_5$ conjugate was tested for its ability to neutralize the infectivity of sporozoites of *P. falciparum* in vitro. The IFA titer of this serum was $10^9$. The neutralization assay was performed as described (16) with two isolates of *P. falciparum* sporozoites and the human hematopoietic passenger HepG2-A16 as the target of parasite invasion. The results (Table 2) showed that the immune immunoglobulin G (IgG) inhibited parasite invasion in a dose-dependent fashion. A strong effect was observed with IgG concentrations as low as 2 µg/ml. When the antibodies to (NANP)$_5$ were removed by absorption with peptide bound to Sepharose beads, the inhibitory effect was abolished (Table 2, experiment 4).

These results strongly suggest that the synthetic peptide (NANP)$_5$ faithfully represents the dominant epitope of the domain of the CS protein containing the NANP repeats. Earlier studies with the *P. knowlesi* CS protein (17, 18) showed that antibodies to its repeat domain also recognize an uninterrupted sequence of amino acids. In *P. falciparum*, the repeat domain contains a large number of prolines and asparagines, residues frequently found in $\beta$-turns (19). Since $\beta$-turns are formed by a few consecutive amino acid residues, the configuration that they

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**Table 2. Neutralization of the infectivity of *P. falciparum* sporozoites in vitro by antibodies to (NANP)$_5$ tetanus toxoid.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number added per culture</th>
<th>Sporozoites</th>
<th>Rabbit immunoglobulin</th>
<th>Origin of serum</th>
<th>Concentration*$^*$</th>
<th>EEF†</th>
<th>Inhibition (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>7G8</td>
<td>$25 \times 10^3$</td>
<td>Preimmune</td>
<td>$2.0$ and $200.0$</td>
<td>$330 \pm 25$</td>
<td>91.2</td>
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<td></td>
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<td>$200.0$</td>
<td>$26, 32$</td>
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<td>$134, 150$</td>
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<tr>
<td>NF54</td>
<td>$17 \times 10^3$</td>
<td>Preimmune</td>
<td>$0.1$ to $100.0$</td>
<td>$264 \pm 18$</td>
<td>68.8</td>
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<td>$240 \pm 9.5$</td>
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<td>$0.2$</td>
<td>$131, 144$</td>
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*Identical concentrations of preimmune and immune IgG were used in every experiment. There were no significant differences in the number of exoerythrocytic forms (EEF) found in hepatoma cells when incubations were performed in the presence of various concentrations of preimmune IgG. EEF number of intracellular (exoerythrocytic) forms. In the case of preimmune sera, the first numbers in each experiment represent the mean ± standard deviation of the EEF observed in coverslips incubated with the various concentrations of nonspecific IgG (4, 12, 10, and 8 coverslips in experiments 1, 2, 3, and 4, respectively); the numbers that follow are the EEF numbers in two coverslips incubated with various concentrations of immune IgG. †Calculated as $(100 - \text{mean experimental values/mean of controls}) \times 100$. (NANP)$_5$, was conjugated to CNBr-Sepharose (Pharmacia) according to the manufacturer's instructions. After conjugation the beads were incubated for 1 hour at 0.25 percent glutaraldehyde, washed, and treated with 1M ethanolamine (pH 8.0) for 2 hours. Equal volumes of beads and immune IgG (0.5 mg/ml) were incubated at room temperature for 60 minutes and the supernatant used in the neutralization assay.
humans, semisynthetic vaccines composed of capsular polysaccharides of pathogenic bacteria covalently bound to carrier proteins have been manufactured and proved effective in man and monkeys (2).

The immunological screening of P. falci- parum sporozoites from different areas of the world showed that all isolates contained representations of the epitope (NANP)_3 (22). Moreover, the (NANP)_3 sequence is repeated in each CS molecule, 37 times in the CS protein from an isolate of P. falciparum from Brazil (7) and at least 23 times in the CS antigen from an isolate from Thailand (8). Because this sequence is so abundantly represented on the surface of the P. falciparum sporozoite, this stage of the parasite should be particularly susceptible to attack by antibodies to (NANP)_3. Synthetic or genetically engineered (NANP)_3-containing antigens are therefore logical candidates for the development of P. falciparum malaria vaccines.

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References and Notes

8. V. Enea et al., ibid. p. 626.
10. Synthetic peptides H(Asn-Ala-Ala-Pro)-H(Asn-Ala-AAs-Pro); H(Asn-Ala-AAs-Pro); H(Asn-Ala-Ala-Pro), were synthesized by the stepwise solid phase method of M. Merrifield (J. Am. Chem. Soc. 85, 2149 (1963)). The attachment of the COOH-terminal residue, as diacid residue, tert-butylglyoxycarbonyl (Boc)-Pro, was onto a hydrogenyl phenylalacetoxy- methyl (PAM) resin (copolyester and 1 per- cent divinylbenzene) support (A. R. Mitchel et al., J. Am. Chem. Soc. 98, 7337 (1976) to prevent loss of peptide chains during the synthesis. BOC-Pro-OCH-Pam-resin (0.4 mmol sub-

Involvement of the bcl-2 Gene in Human Follicular Lymphoma

Abstract. Recombinant DNA probes were cloned for the areas flanking the breakpoint on chromosome 18 in cells from a patient with acute lymphocytic leukemia of the B-cell type; cells of this line carry the t(4;18) chromosomal translocation. Two of the probes detected DNA rearrangements in approximately 60 percent of the cases of follicular lymphoma screened. In follicular lymphoma, most of the breakpoints in band q21 of chromosome 18 were clustered within a short stretch of DNA, approximately 2.1 kilobases in length. Chromosome-specific DNA probes also flanked the breakpoint in the DNA transcribed into 2.1 kilobases in length in various cell types. The gene coding for these transcripts (the bcl-2 gene) seems to be interrupted in most cases of follicular lymphomas carrying the t(14;18) chromosomal translocation.

Follicular lymphoma is one of the most common human B-cell neoplasms; in most patients the lymphoma cells carry a translocation between chromosomes 14 and 18 (1,2). By taking advantage of an established cell line, 380, derived from a patient with acute lymphocytic leukemia of the B-cell type (3), we cloned the DNA region joining chromosomes 14 and 18 on the 14q chromosome of line 380 cells (4). Using chromosome 18-specific DNA probes flanking the chromosome breakpoint of line 380 cells, we also showed DNA rearrange-ments of the homologous DNA segments in follicular lymphoma cells with the t(14;18) chromosome translocation (4).

In the present study we have “walked” on the region of chromosome 18 that is involved in chromosome rearrangements in B-cell neoplasms to map the breakpoints in follicular lymphoma and to identify the putative bcl-2 gene, which may harbor the translocation. In non-malignant follicular lymphoma and other B-cell neoplasms carrying the t(14;18) chromo-
some translocation.

By starting with DNA probes that mapped close to the t(14;18)-associated breakpoint on chromosome 18 in line 380 leukemic cells, we were able to “walk” on chromosome 18 in both directions and

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