The Moran Foundation
DEPARTMENT OF PATHOLOGY
BAYLOR COLLEGE OF MEDICINE
TEXAS MEDICAL CENTER
HOUSTON, TEXAS 77030

June 23, 1992

Gary K. SeGall, M.D., Ph.D.
Department of Pathology
The Methodist Hospital

Dear Dr. SeGall:

Please update me on the status of your Moran Foundation project (3-90-0048) entitled "Human Herpes Virus 6 in Primary CNS Lymphoma".

Since approval and funding is generally for a one-year period, all projects approved in or prior to June 1991 should now be "complete", or nearly so.

I need a progress and/or final report regarding your project, including dates and times of any presentations, and information regarding any publications.

Please submit this to me within the next 30 days.

Sincerely yours,

Philip J. Migliore, M.D.
Research Director

PJM/ms

c: Dr. Michael Lieberman
Mr. John Moran

THE METHODIST HOSPITAL
Pathology Service
6565 Fannin, M.S. 205
Houston, Texas 77030

BAYLOR COLLEGE OF MEDICINE
Department of Pathology
One Baylor Plaza
Houston, Texas 77030
(713) 798-4661

Houston, July 7, 1992

Philip J. Migliore, MD
Research Director
Moran Foundation

Re: Project 3-90-0048

Dear Dr. Migliore:

The following is a summary of the activities related to the above project.

HHV-6 in central nervous system lymphomas

This was the main focus of the project and also unfortunately quite a disappointment. We examined DNA from 27 primary CNS lymphomas for the presence of HHV-6, HIV, and EBV genome using PCR. Results for EBV were inconsistent and not reproducible. One case was positive for both HIV and HHV-6 sequences and this was confirmed repeatedly. In-situ hybridization to detect HHV-6 and HIV mRNA was uninterpretable due to high background levels and immunohistochemistry to detect viral antigens was repeatedly negative. The tumor was removed from a 47-year old woman who is alive and well without evidence of tumor or HIV-related disease almost two years after the resection. It is very difficult to make sense of these results and we are not going to attempt to publish them.

Clonality analysis in lymphoid lesions using PCR

This was done as a secondary project but turned out to be quite worthwhile. We used a PCR-based method to detect rearrangements of the immunoglobulin heavy chain genes and applied it to 23 of our lymphomas. Clonal rearrangements could be demonstrated in a number of them. Of interest is the fact that this was performed on DNA extracted from paraffin blocks; this method thus allows clonality analysis in archival material that is not suitable for conventional clonality analysis (Southern blot and light chain restriction). The work was presented as an abstract at the meeting of the American Association for Cancer Research in San Diego, May 20-23, 1992. It has also been accepted for publication in the JOURNAL OF PATHOLOGY. A copy of the revised manuscript is included.

THE METHODIST HOSPITAL
Pathology Service
6565 Fannin, M.S. 205
Houston, Texas 77030

BAYLOR COLLEGE OF MEDICINE
Department of Pathology
One Baylor Plaza
Houston, Texas 77030
(713) 798-4661

In a similar vein, we used the method to demonstrate restricted clonality in an atypical pulmonary plasma cell infiltrate, again using DNA extracted from paraffin blocks. This was presented as an abstract in April 1992 at the Housestaff Research Symposium organized by Baylor's Dept. of Medicine and won the prize for best clinical paper (Subramanian D, Gonzalez JM, Albrecht S, Cagle PT. Pulmonary infiltrates and macroglobulinemia). A manuscript for publication is in preparation.

We wish to thank the Moran Foundation for their generous support of our project.

Sincerely yours,

Gary K. SeGall

G.K. SeGall, PhD, MD
Dept. of Pathology
The Methodist Hospital

Dr Meglasson,

- I am sending the originals by post. I hope this is enough for the meeting.

Thanks,

Mary

Immunoglobulin heavy chain rearrangements in primary brain lymphomas
A study using PCR to amplify CDR-III

REVISED MANUSCRIPT

Steffen Albrecht, MD,* Janet M. Bruner, MD,** Gary K. SeGall, PhD, MD*

Depts. of Pathology, Baylor College of Medicine* and M.D. Anderson Cancer Center,** Houston, TX

Running title: **CDR-III PCR**

Address for correspondence:

G.K. SeGall, PhD, MD
Dept. of Pathology
Northside Medical Center
500 Gypsy Lane
Youngstown
Ohio 44501

(This work was presented in abstract form at the meeting of the American Association for Cancer Research in San Diego, May 20-23, 1992.)

ABSTRACT

Primary brain lymphomas (PBL) have only rarely been analyzed for immunoglobulin heavy chain (IgH) rearrangements. In this study, DNA was extracted from paraffin blocks in 23 cases of PBL and examined for IgH rearrangements using the polymerase chain reaction (PCR) to amplify the complementarity-determining region III (CDR-III) of rearranged IgH genes. Fifteen of the cases were phenotyped on paraffin embedded tissue using a pan-B and a pan-T antibody (L26 and UCHL-1, respectively). The remaining 8 cases were not phenotyped for lack of tissue. For comparison, we used DNA extracted from paraffin blocks of normal brain, lymph nodes with lymphoid hyperplasia, and non-lymphoid malignancies. PCR products were examined by polyacrylamide gel electrophoresis. Among the 10 B-cell PBL, 4 had a pattern indicative of IgH rearrangement, 1 had a germline pattern, and 5 had no detectable PCR products. Among the 5 T-cell PBL, 1 had a germline pattern, and 4 had no detectable products. Among the 8 untyped PBL, 2 had IgH rearrangement, 4 had a germline pattern and 2 gave no detectable products. DNA from non-lymphoid tissues gave a consistent germline pattern while DNA from polyclonal lymphoid populations (lymph node) had a pattern of polyclonal IgH rearrangement. In a dilution study, a clonal rearrangement could be detected as long as the clone's DNA constituted at least 10% of the total DNA. PCR to amplify CDR-III can be successfully applied to DNA extracted from paraffin blocks and detected a clonal rearrangement in 50% of cases that gave a detectable pattern. This allows clonality analysis of tissue unsuitable for conventional Southern blot analysis. Furthermore, B-cell PBL have IgH rearrangements similar to those of extracranial B-cell neoplasms.

Key words: immunoglobulin heavy chain, gene rearrangement, PCR, CDR-III, primary brain lymphoma

Introduction

B-lymphocytes rearrange their immunoglobulin heavy chain (IgH) genes early in the course of their maturation. The rearranged gene has the following organization: (5')V_H-N_1-D_H-N_2-J_H(3') where V_H, D_H, and J_H are the variable, diversity, and joining segments, respectively. The "N" sequences are random seqences created during the V_H-D_H and D_H-J_H joinings through random insertion of nucleotides by the enzyme terminal deoxynucleotide transferase (TdT) The region lying between the V_H and J_H segments is known as "complementarity-determining-region III" (CDR-III) and is specific to a given lymphocyte and its clone of descendents. Since both the V_H and J_H segments contain conserved regions it is possible to amplify the CDR-III selectively using the polymerase chain reaction (PCR) with primers complentary to these conserved flanking regions (1,2).

Examination of the CDR-III PCR products by gel electrophoresis gives an indication of the clonality of the lesion (3). These products are ≈ 100 - 200 base pairs (bp) in length. In a monoclonal B-cell population, there will be only 1 or 2 CDR-III (depending on whether only one or both of the IgH alleles are rearranged) and on the gel, each will appear as a distinct, narrow band. In a polyclonal B-cell population, there are numerous lymphoid clones, each with its own band somewhere in the range of 100 - 200 bp. On a gel, these will add up to a broad smear or smudge.

We wanted to determine whether this method could be applied to DNA extracted from paraffin-embedded lymphoid lesions, since such material is not suitable for either Southern blot or light chain restriction analysis. Furthermore, we wanted to examine how the presence of non-lymphoid germline DNA influenced the results of this method. We therefore used PCR amplification of CDR-III to study the clonality of primary brain lymphomas (PBL) on which only paraffin blocks were available.

Material and Methods

A total of 23 cases of PBL were located in the files of the pathology departments of The Methodist Hospital and the M.D. Anderson Cancer Center for the period from 1978 to 1991. One case was an autopsy case, the others were surgical specimens (mostly biopsies). In the patients that had only

been biopsied, subsequent staging work-up had not shown evidence of extracranial lymphoma. No patient was known to have AIDS. All tissues had been fixed in 10% neutral formalin, routinely processed and embedded in paraffin. No frozen tissue was available. All tumors had a diffuse histological pattern and were of a large cell type.

Immunohistochemistry: Immunophenotyping was performed on paraffin sections. A two step indirect avidin-biotin method was used. The L26 antibody (Dako) served as a pan-B cell marker (4) and the UCHL-1 antibody (Dako) as a pan-T cell marker (5). Sections were not predigested. Dilutions were 1:300 and 1:500 respectively. The sections were incubated overnight at 4°C. Detection was with the ABC kit (Vector). Aminoethylcarbazole was used as chromogen. Paraffin sections of similarly processed lymph nodes were used as positive controls. The primary antibody was omitted for negative controls.

DNA extraction: DNA was extracted from paraffin blocks from 23 PBL, 5 hyperplastic lymph nodes, 5 non-lymphoid malignancies (2 breast carcinomas, 1 colon carcinoma, 1 lung carcinoma and 1 glioblastoma multiforme) and 1 cerebellum obtained at autopsy. Depending on the amount of tissue, from 2 to 12 sections were cut at 30 µm from a representative block of each lesion. The sections were deparaffinized with xylene and rehydrated through graded alcohols. This was followed by 1 wash in distilled water and 3 washes in PBS. After the final wash, the sections were minced if necessary to reduce the size of the tissue fragments to about 3-4 mm. The tissue was the treated with proteinase K (Sigma) at 200 µg/ml in a buffer containing 10 mM Tris-HCl (pH = 8), 10 mM EDTA, 10 mM NaCl and 0.5 % SDS. Digestion was performed at 37°C with agitation for 16-24 h. DNA was extracted with phenol followed by a 25:24:1 (v/v) mixture of phenol:chloroform:isoamyl alcohol (PCI). The resulting aqueous extract was dialysed overnight at 4°C against a buffer containing 50 mM Tris-HCl (pH = 8), 10 mM EDTA and 10 mM NaCl. The dialysate was then treated with ribonuclease A (Sigma) at 50 µg/ml for 3-4 h at 37°C with agitation. Proteinase K and SDS were then added to a final concentration of 50 µg/ml and 1 % respectively and incubation was continued overnight. The DNA was again extracted with phenol and PCI and the final aqueous phase was dialysed for several hours at 4°C against a buffer

containing 10 mM Tris-HCl (pH = 8), 0.5 mM EDTA and 10 mM NaCl. The dialysate was concentrated by placing the dialysis tubes on a bed of polyethylene glycol until they became flaccid. The dialysis was then continued in a fresh change of the same buffer overnight. DNA concentration was estimated by measuring absorbance at 260 nm. The final extracts were stored at 4°C.

PCR protocol: We used the method of McCarthy et al (3). The sequences (5′ to 3′) of the primers were CTG TCG AGA CGG CCG TGT ATT ACT G for the V_H segment and AAC TGC AGA GGA GAC GGT GAC C for the J_H segment. The first primer recognizes the 3′ end of the so-called FR3 region of V_H segments while other primer recognizes the 3′ end of J_H segments (1). PCR was performed in a final volume of 100 µl as described previously (3) without any modification of the original technique. Each run included a negative control consisting of PCR mixture with no DNA other than the primers.

PCR products were analysed on 10% polyacrylamide gels (0.75 mm thickness) in 1 X TBE running buffer (45 mM Tris-borate and 1 mM EDTA, ph = 8.3). Per well, 5 µl of PCR reaction mixture were loaded. Electrophoresis was carried out on a Miniprotean-II apparatus (BioRad) for 70 min at 150 V. The gels were then stained with ethidium bromide at 1µg/ml for 15 min and photographed under ultraviolet light. As size markers, we used *Hae*III fragments of FX174 RF (Gibco-BRL) which contain 11 bands ranging from 72 to 1353 bp.

Results

Immunohistochemistry: When evaluating staining, we only considered large, atypical appearing lymphoid cells. Small, mature lymphocytes were considered to be reactive. The slides were read without knowledge of the PCR results. Of the 15 phenotyped tumors, 10 were positive with the L26 antibody, indicating B-cell lineage. The remaining 5 labelled with the UHCL-1 antibody, indicating T-cell lineage.

PCR: The PCR results are summarized in Table I. Germ line DNA from non-lymphoid malignancies and normal cerebellum gave a complex pattern with approximately 20 bands concentrated in the 300-1300 bp region but extending down to ≈ 100 bp. The banding pattern was identical among the different samples, although corresponding bands did not necessarily have the same

intensity (fig. 1). The 5 hyperplastic lymph nodes had a polyclonal pattern with a smudge around 100-150 bp (fig. 2).

Four of the 10 B-cell PBL had a clonal pattern (fig. 3 and fig. 4, lanes 1-2). Two of these had a single dominant band at ≈ 100 bp and 1 had a single band at ≈ 200 bp. In the 4th case, no bands were seen in the 100-200 bp range, but there was a strong band at ≈ 500 bp and another one at ≈ 1100 bp. Among the 6 remaining B-cell tumors, 1 had a germline pattern and the other 5 had no detectable PCR products.

Among the 5 known T-cell lymphomas, none had a clonal pattern. One case gave a germline pattern and the remaining 4 had no detectable products.

Of the 8 lymphomas that had not been typed, 2 had clonal pattern with a dominant band at ≈100 bp (fig. 4, lanes 3 - 6), 4 had a germline pattern and 2 gave no detectable products.

In those lymphomas that did not yield detectable products after one round of PCR, we subjected 10 µl of the PCR products to a second round of amplification. This did yield visible bands; however, these were not reproducible on subsequent runs, indicating that they represent random secondary PCR products.

To test the sensitivity of the method, DNA from a lymphoma with a well defined band was mixed with normal cerebellar DNA so that it constituted 50, 20, 10, 5, 1, and 0% of the total DNA. The band produced by the lymphoma could be detected easily down to 10% and was still faintly visible at 5% (fig. 5).

The negative controls (e.g., PCR mixture containing no DNA other than the primers) gave no detectable pattern when analysed by gel electrophoresis (data not shown).

Discussion

PCR amplification of the CDR-III of rearranged IgH genes was initially developed as a sensitive tool for the detection of residual disease in patients with B-cell malignancies (1,2). In a tumor with a clonal IgH rearrangement, the CDR-III can be amplified and sequenced. A clone-specific oligonucleotide probe is then synthesized and used to detect neoplastic DNA in tissue samples

with a sensitivity of 0.1% (2). The method has also been applied to DNA extracted from archival bone marrow smears (6).

However, amplification of CDR-III by PCR can also be used to assess clonality in B-cell proliferations (3). McCarthy et al studied 10 cases of B-cell lesions (with IgH rearrangements demonstrated by Southern blot analysis) using DNA extracted from fresh or frozen tissue. Using the same method as employed in this paper, PCR gave evidence of clonality in 8 of the 10 cases. CDR-III PCR does not detect all rearrangements because although the JH primer recognizes all 6 JH segments, the VH primer recognizes most but not all of the several hundred VH segments (3,7). Sensitivity was 5% (3). In another study using a similar though not identical approach, PCR detected clonal IgH rearrangements in 19 of 23 B-cell lesions (82%) using DNA extracted from fresh frozen tissues (8). With a single VH primer, the maximum rate of detection of clonality would appear to be ≈ 80%. More recently, a rate of > 90% was achieved by using a "cocktail" of 7 VH primers that recognize the FR1 region of the VH gene rather than the FR3 region (7).

In our study, DNA was extracted from paraffin blocks, not from fresh tissue, and the tumors were unselected with regard to their IgH rearrangement status. More than 90% of lymphomas with a B-cell phenotype have Ig gene rearrangements (9). It is thus doubtful that our cases of PBL that didn't yield detectable PCR products failed to do so because they lacked such rearrangements. Some cases may have had rearrangements that cannot be detected by our method (see above). In others, the germline pattern produced by contaminating brain DNA may have obscured a band stemming from the lymphoma. Furthermore, DNA extracted from paraffin blocks tends to be degraded and may not amplify.

Nevertheless, we were able to demonstrate evidence of an IgH rearrangement in a number of cases using material that would not have been suitable either for immunohistochemistry (to demonstrate light chain restriction) or for Southern blot since surface immunoglobulins are denatured by formalin fixation and DNA obtained from paraffin blocks is too degraded for Southern analysis. Obviously, we are not advocating the use of CDR-III PCR amplification on paraffin tisue as a routine method to replace the other two techniques applied to fresh or frozen tissue. However, in lesions such as PBL, where there is often no such tissue available, CDR-III PCR can yield valuable information about clonality.

(One interesting and somewhat puzzling result of our study is the presence of a distinct and reproducible banding pattern when non-lymphoid DNA is analyzed. The non-rearranged IgH segments are several thousand bp apart and the taq polymerase cannot efficiently bridge such large distances between primers. This pattern might result from interaction with the primers and other genes of the Ig superfamily. When PCR was performed on mixtures containing no DNA other than the primers, no pattern was observed. This indicates that the pattern obtained with genomic DNA is not merely the result of primer-primer interactions. The other unusual result is the presence of very large PCR products in one of our B-cell PBL. It has recently been shown that some IgH rearrangements are more complex than the usual V_H-D_H-J_H scheme. In particular, there are V_H-D_H-D_H-J_H rearrangements and rearrangements with long "N" sequences (10,11). These lead to a CDR-III that is considerably longer than normal.)

Conventional Southern blot analysis of IgH rearrangements requires ≈10 µg of high molecular weight DNA extracted from fresh or frozen tissue. It also requires restriction enzyme digestion of the DNA and the use of radioactive probes. The whole analysis takes several days. Under ideal conditions, sensitivity is about 1% (12) but some authors consider a sensitivity of 5-10% more realistic in a clinical laboratory setting (13). PCR for CDR-III requires less DNA, does not require radioactivity and can be done in about 4 h (including the running time for the gel).

PBL have a similar morphology and express the same lymphocyte markers as those of extracranial lymphomas with most having a B-cell phenotype (14). Ig gene rearrangements in PBL have only rarely been studied (15,16). In these two reports, a total of 10 cases were analyzed. All had a B-cell immunophenotype. IgH rearrangements were present in all cases. Nine cases were also analyzed for light chain gene rearrangements which were present in 8. Our results are in agreement with these studies. Taken together, all these reports underscore that PBL are true lymphomas not only histologically and immunophenotypically, but also genotypically.

In summary, our findings indicate that PCR for CDR-III to analyse IgH rearrangements can be applied with some success and with reasonable sensitivity to DNA extracted from routine histological material. The method could be used for retrospective analysis of lymphoid lesions for which no

fresh or frozen tissue is available. In the case of B-cell PBL, it demonstrates IgH rearrangements similar to those of extracranial B-cell lymphomas.

Acknowledgement

This study was supported by the Moran Foundation (Houston, TX; # 3-90-0048)

References

1. Yamada M, Hudson S, Tournay O, *et al.* Detection of minimal disease in hematopoietic malignancies of the B-cell lineage by using third-complementarity-determining region (CDR-III)-specific probes. *Proc Natl Acad Sci USA* 1989; **86**: 5123-5127.

2. Yamada M, Wasserman R, Lange B, Reichard BA, Womer RB, Rovera G. Minimal residual disease in childhood B-lineage lymphoblatic leukemia. Persistence of leukemic cells during the first 18 months of treatment. *N Eng J Med* 1990; **323**: 448-455.

3. McCarthy KP, Sloane JP, Widemann LM. Rapid method for distinguishing clonal from polyclonal B cell populations in surgical biopsy specimens. *J Clin Pathol* 1990; **43**: 429-432.

4. Davey FR, Elghetany MT, Kurec AS. Immunophenotyping of hematologic neoplasms in paraffin-embedded tissue sections. *Am J Clin Pathol* 1990; **93(Suppl 1)**: S17-S26.

5. Cabeçadas JM, Isaacson PG. Phenotyping T-cell lymphomas in paraffin sections - which antibodies? *Histopathology* 1991; **19**: 419-424.

6. Asada M, Miyashita T, Bessho F, Kobayashi N, Mizutani S. Malignant cell detection in Burkitt's lymphoma using third-complementarity determining region (CDRIII), clone-specific probe developed by sequencing DNA from stored slides. *Jpn J Cancer Res* 1991; **82**: 848-853.

7. Deane M, McCarthy KP, Wiedemann LM, Norton JD. An improved method for detection of B-lymphoid clonality by polymerase chain reaction. *Leukemia* 1991; **5**: 726-730.

8. Trainor KJ, Brisco MJ, Story CJ, Morley AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. *Blood* 1990; **75**: 2220-2222.

9. Rudders RA, Dhillon S, Krontiris TG. Clonal analysis of untreated non-Hodgkin's lymphoma utilizing immunoglobulin gene rearrangement and immunophenotype. *Cancer Res* 1988; **48**: 6272-6277.

10. Yamada M, Wasserman R, Reichard BA, Shane S, Caton AJ, Rovera G. Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult human peripheral blood lymphocytes. *J Exp Med* 1991; **173**: 395-407.

11. Price CGA, Whitehouse JMA. Complex D_H-J_H junctions in normal human lymphocytes (abstr). *Proc Am Assoc Cancer Res* 1992; **33**: 250.

12. Sklar J. What can DNA rearrangements tell us about solid hematolymphoid neoplasms? *Am J Surg Pathol* 1990; **14(Suppl 1)**: 16-25.

13. Willman CL, Griffith BB, Whittaker M. Molecular genetic approaches for the diagnosis of clonality in lymphoid neoplasms. *Clin Lab Med* 1990; **10**: 119-149.

14. Grant JW, Isaacson PG. Primary central nervous system lymphoma. *Brain Pathol* 1992; **2**: 97-109.

15. Smith WJ, Garson JA, Bourne SP, Kemshead JT, Coakham HB. Immunoglobulin rearrangement and antigenic profile confirm B cell origin in primary cerebral lymphoma and indicate a mature phenotype. *J Clin Pathol* 1988; **41**: 128-132.

16. Kumanishi T, Washiyama K, Nishiyama A, Abe S, Saito T, Ichikawa T. Primary malignant lymphoma of the brain: demonstration of immunoglobulin gene rearrangements in four cases by the Southern blot hybridization technique. *Acta Neuropathol (Berl)* 1989; **79**: 23-26.

TABLE I

Results of CDR-III PCR

Tissue (number of cases)	Polyclonal rearrangement	Clonal rearrangement	Germline pattern	No detectable PCR products
non-lymphoid malignancies (5)	0	0	5	0
hyperplastic lymph nodes (5)	5	0	0	0
B-cell PBL (10)	0	4	1	5
T-cell PBL (5)	0	0	1	4
untyped PBL (8)	0	2	4	2

PBL: primary brain lymphoma

LEGENDS

Figure 1: Polyacrylamide gel electrophoresis (PAGE) of CDR-III PCR products from non-lymphoid malignancies and brain: (1) colon carcinoma, (2) lung carcinoma, (3) and (4) breast carcinoma, (5) blank, (6) gliosarcoma, (7) normal cerebellar DNA, (S) DNA size marker (bp).

Figure 2: PAGE of CDR-III PCR products from hyperplastic lymph nodes: (1) - (5) lymph nodes, (S) DNA size marker (bp).

Figure 3: PAGE of CDR-III PCR products from primary brain lymphomas: (1) normal cerebellar DNA, (2) - (7) paired samples from 3 lymphomas immunophenotyped as B-cell lesions, (S) DNA size marker (bp).

Figure 4: PAGE of CDR-III PCR products from primary brain lymphomas: (1) - (6) paired samples from 3 lymphomas (the case in lanes 1 and 2 had been immunophenotyped as a B-cell lesion while the 2 other cases could not be typed), (7) normal cerebellar DNA, (S) DNA size marker (bp).

Figure 5: PAGE of CDR-III PCR products from dilution study in which lymphoma DNA was mixed with cerebellar DNA. The lymphoma is the same lesion as that in lanes 3 and 4 of fig. 4. The numbers across the top indicate the percentage of lymphoma DNA. (S) DNA size marker (bp).