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Unique Forms of the *abl* Tyrosine Kinase Distinguish Ph¹-Positive CML from Ph¹-Positive ALL

STEVEN S. CLARK, JAMI MCLAUGHLIN, WILLIAM M. CRIST, RICHARD CHAMPLIN, OWEN N. WITTE*

In the Philadelphia chromosome (Ph¹) of chronic myelogenous leukemia (CML), the c-*abl* gene on chromosome 9 is translocated to *bcr* on chromosome 22. This results in the expression of a chimeric *bcr-abl* message that encodes the P210$^{bcr\text{-}abl}$ tyrosine kinase. The cells of 10% of acute lymphocytic leukemia patients (ALL) carry a cytogenetically similar Ph¹ translocation. We report that Ph¹-positive ALL cells express unique *abl*-derived tyrosine kinases of 185 and 180 kilodaltons that are distinct from the *bcr-abl*–derived P210 protein of CML. The appearance of the 185/180-kilodalton proteins correlates with the expression of a novel 6.5-kilobase messenger RNA. Thus, similar genetic translocations in two different leukemias result in the expression of distinct c-*abl*–derived products.

THE LEUKEMIC CELLS OF MORE than 90% of chronic myelogenous leukemia (CML) patients carry the t(9;22) (q34;q11) that generates the Philadelphia chromosome (Ph¹) (*1*). One molecular consequence of Ph¹ is the translocation of the c-*abl* oncogene on chromosome 9 into a 5.8-kb breakpoint cluster region in the *bcr* gene (*2*). Although the breakpoints on both chromosomes are variable, RNA-splicing generates a unique 8-kb *bcr-abl* chimeric message that is larger than the normal 6- and 7-kb c-*abl* transcripts seen in most tissues (*3, 4*). This 8-kb message encodes a phosphoprotein (P210$^{bcr\text{-}abl}$) that contains *bcr*-encoded NH_2-terminal sequences and a c-*abl*–derived COOH-terminal segment (*5*). The structure of P210$^{bcr\text{-}abl}$ is reminiscent of the P160$^{v\text{-}abl}$ transforming protein encoded by the *gag-abl* gene of Abelson murine leukemia virus. As a consequence of NH_2-terminal structural alteration, both P210$^{bcr\text{-}abl}$ and P160$^{v\text{-}abl}$ have similar tyrosine kinase activities that are distinct from the activity of the normal c-*abl* product, P145$^{c\text{-}abl}$ (*6*). In the case of P160$^{v\text{-}abl}$, the tyrosine-specific kinase activity is essential for cellular transformation (*7*). By analogy, P210$^{bcr\text{-}abl}$ tyrosine kinase activity may play an important part in the pathogenesis of CML.

P210$^{bcr\text{-}abl}$ expression may be considered diagnostic of Ph¹-positive CML since this protein is found in a wide range of Ph¹-positive CML cell lines and patient samples (*8, 9*). Ph¹ is also found in approximately 10% of patients with human acute lymphocytic leukemias (ALL) and is indistinguishable from the Ph¹ of CML by cytogenetics (*10*). In contrast to CML, the breakpoint cluster region is not involved in the translocation in at least some Ph¹-positive ALL patients (*11*). We reasoned, therefore, that if altered c-*abl* expression occurs without the usual breakpoint cluster rearrangement in Ph¹-positive ALL, the molecular mechanism of expression would be different from that in Ph¹-positive CML. This could result in the expression of a novel c-*abl* product whose structure and/or function would be characteristic of Ph¹-positive ALL and distinct from Ph¹-positive CML.

By means of site-directed rabbit antisera specific for different regions of the v-*abl* protein (*12*), we examined c-*abl* protein expression in the blood and bone marrow of several CML and ALL patients (*13*). The c-*abl* proteins were precipitated with the immune sera but not with the preimmune control antisera (Fig. 1, lanes 1 and 2). Autophosphorylation of P210$^{bcr\text{-}abl}$ and, to a lesser extent, P145$^{c\text{-}abl}$, was detected in the CML-derived K562 cell lysates (Fig. 1A) as

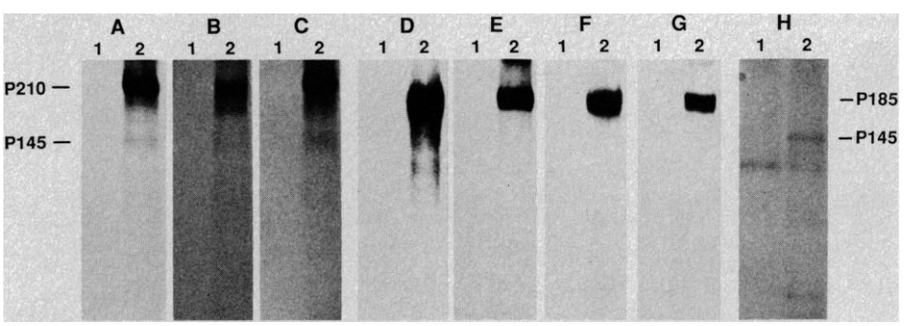

Fig. 1. In vitro kinase analysis of Ph¹-positive CML and Ph¹-positive ALL *abl* proteins. Ph¹-positive CML proerythroblastic cell line K562 (**A**); Ph¹-positive CML blast-crisis patients 146-03-37 (**B**) and 143-57-16 (**C**); Ph¹-positive ALL patients 9556 (**D**), 045-14-02 (**E**), 9254 (**F**), and 9312 (**G**); and a Ph¹-negative ALL patient 9913 (**H**). Patient samples were thawed and diluted into 15 ml of RPMI containing 10% fetal bovine serum, then incubated for 2 to 4 hours in CO_2 at 37°C. Cells were then washed and lysed in kinase lysis buffer (KLB) (*6*). Lysates were immunoprecipitated overnight with normal rabbit sera (lane 1) or with rabbit antisera anti-pEX5 specific for the COOH-terminal region of v-*abl* (lane 2) (*12*). Immune complexes were collected on protein A–Sepharose (Pharmacia) and kinase reactions were run in the presence of 20 μCi of ^{32}P-labeled adenosine triphosphate for 30 minutes at 30°C to get maximal autophosphorylation (*6*). Reactions were terminated by washing twice in KLB plus 5 m*M* EDTA. Precipitated proteins were recovered by boiling in sample buffer and diluted 20-fold in KLB according to Konopka *et al.* (*5*). Samples were reprecipitated with antisera specific for the *abl* kinase domain (anti-pEX2) (*12*), collected on protein A–Sepharose, then analyzed by 8% SDS–polyacrylamide gel electrophoresis followed by autoradiography. Approximate sizes of radiolabeled proteins were determined by comparison with nonradioactive, prestained high molecular weight standards (BRL). Exposure times were for 2 to 24 hours in panels A to G and for 14 days in panel H.

S. S. Clark, J. McLaughlin, O. N. Witte, Department of Microbiology and Molecular Biology Institute, University of California, Los Angeles, CA 90024.
W. M. Crist, Department of Hematology/Oncology, St. Jude Children's Research Hospital, Memphis, TN 38101, and Department of Pediatrics, University of Tennessee, College of Medicine, Memphis, TN.
R. Champlin, Department of Medicine, Division of Hematology/Oncology, UCLA Center for the Health Sciences, Los Angeles, CA 90024.

*To whom correspondence should be addressed.

previously reported (*5*). P210$^{bcr\text{-}abl}$ autokinase activity has been found in a variety of CML-derived cell lines, but has not been demonstrated in clinical isolates from CML patients. Two blast-crisis stage CML patient samples, previously shown by ^{32}P-orthophosphate labeling to express P210$^{bcr\text{-}abl}$ (*14*) were analyzed for P210$^{bcr\text{-}abl}$ autokinase activity. We found that CML patient samples express P210$^{bcr\text{-}abl}$ amd P145$^{c\text{-}abl}$ autokinase activity (Fig. 1, B and C). For controls, cells from a Ph^1-negative myeloproliferative syndrome patient and the promyelocytic HL60 cells were examined and only showed P145$^{c\text{-}abl}$ autokinase activity (*14*).

In samples from four Ph^1-positive ALL patients, strong in vitro autokinase activity was seen in a region of about 185 kD referred to as P185$^{all\text{-}abl}$ (Fig. 1, D to G), which could be resolved into two closely migrating bands of approximately 185 kD and 180 kD (Fig. 1, F and G). These may represent different translational products or posttranslational modification of a single product. We did not detect P145$^{c\text{-}abl}$ autokinase activity in the Ph^1-positive ALL cells. Nor did we detect P145$^{c\text{-}abl}$ in immunoprecipitated lysates of ^{32}P-orthophosphate–labeled Ph^1-positive ALL cells (*14*). When large numbers of Ph^1-negative ALL cells were tested in the kinase assay, low levels of P145$^{c\text{-}abl}$ were found (Fig. 1H). It is possible that P145$^{c\text{-}abl}$ is expressed at low levels in lymphoid cells and its detection may actually reflect the presence of non-lymphocytic cells in the samples. Alternatively, elevated P185$^{all\text{-}abl}$ expression may exert a feedback signal on P145$^{c\text{-}abl}$ expression in the leukemic cells. This pattern of autokinase activity is a general characteristic of Ph^1-positive ALL since it was shown in six of seven Ph^1-positive ALL patients but not in three Ph^1-negative patients (Fig. 1) (*14*).

The in vitro kinase activity of P145$^{c\text{-}abl}$, P160$^{v\text{-}abl}$, and P210$^{bcr\text{-}abl}$ have all been shown to be tyrosine-specific (*6*). To determine the kinase specificity of P185$^{all\text{-}abl}$, proteins labeled in vitro were partially acid-hydrolyzed and examined by thin-layer electrophoresis. P210$^{bcr\text{-}abl}$ proteins isolated from K562 cells phosphorylate on tyrosine (Fig. 2A). Similarly, P185$^{all\text{-}abl}$ from three different Ph^1-positive ALL patients also show tyrosine-specific autophosphorylation (Fig. 2, B to D).

The relationship of the various c-*abl* proteins detected in CML and ALL patients was further compared by two-dimensional complete tryptic phosphopeptide mapping of in vitro phosphorylated proteins (Fig. 3). P210$^{bcr\text{-}abl}$ proteins obtained from either K562 cells or from fresh clinical isolates generated similar phosphopeptide maps (Fig. 3, A and B). Both P210$^{bcr\text{-}abl}$ samples autophosphorylated the same two or three major sites and several minor sites. In contrast, P145$^{c\text{-}abl}$ phosphorylates a single major site in the kinase domain (*6*) that is distinct from P210$^{bcr\text{-}abl}$ phosphorylation sites (Fig. 3C). The ALL P185$^{all\text{-}abl}$ showed a single major autophosphorylation site and about three minor sites (Fig. 3D). The major phosphorylated site of P185$^{all\text{-}abl}$ was very similar to the P145$^{c\text{-}abl}$ autophosphorylation site and was clearly distinct from the P210$^{bcr\text{-}abl}$ and P160$^{v\text{-}abl}$ sites (*14*).

To identify the basis for P185$^{all\text{-}abl}$ expression in Ph^1-positive ALL samples, we compared the structure of *abl* messenger RNA (mRNA) in Ph^1-positive ALL and

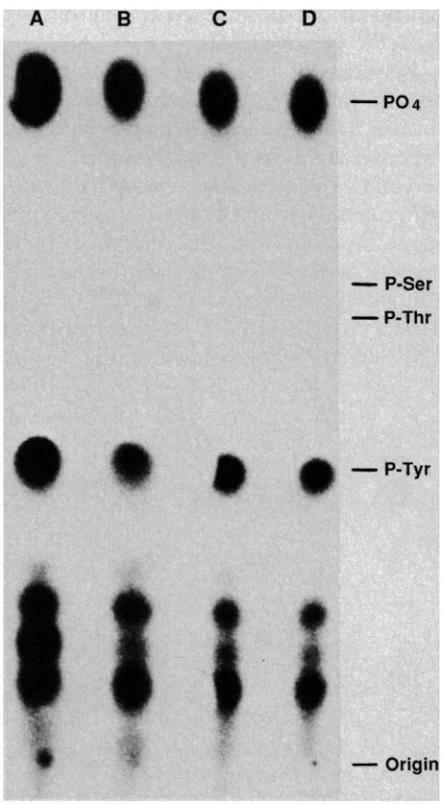

Fig. 2. Phosphoamino acid analysis of *abl* proteins phosphorylated in vitro P210$^{bcr\text{-}abl}$ from K562 cells (**A**) and P185$^{all\text{-}abl}$ from Ph^1-positive ALL patients 9254 (**B**), 9453 (**C**), and 045-14-02 (**D**) were autophosphorylated as described in Fig. 1. Proteins were eluted from the gel and partially hydrolyzed with 4*N* HCl (*6*). Each sample (approximately 1000 count/min) was separated by electrophoresis on a thin-layer cellulose plate and phosphoamino acid composition was detected by autoradiography for 5 days. Nonradioactive standards were detected by ninhydrin staining (*14*).

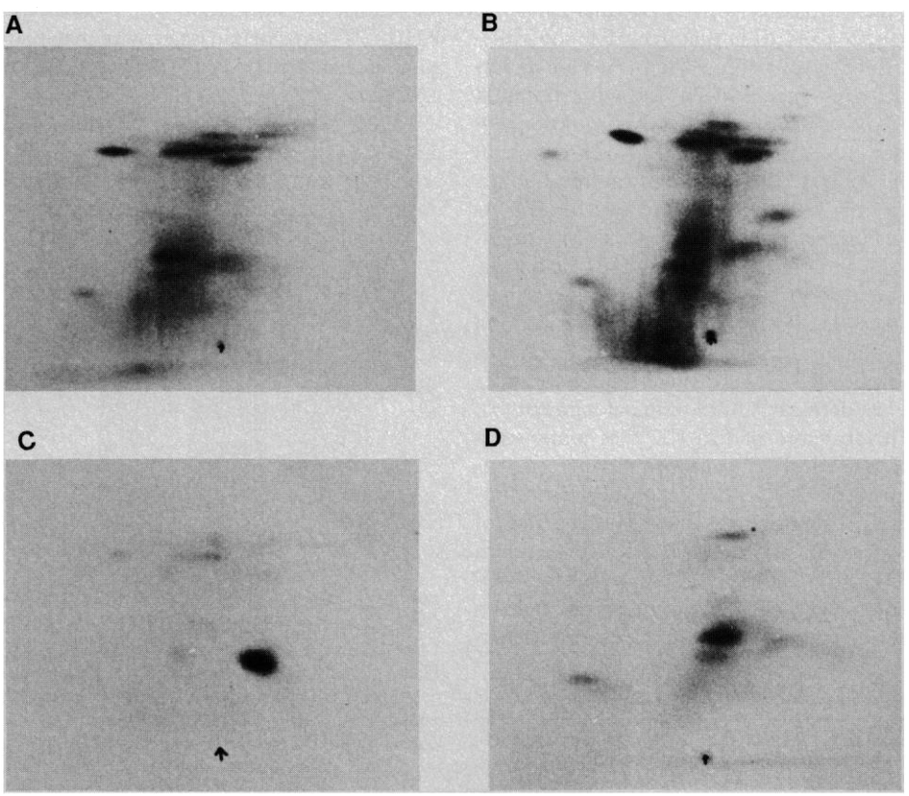

Fig. 3. Two-dimensional phosphopeptide analysis of in vitro phosphorylated *abl* proteins. P210$^{bcr\text{-}abl}$ from K562 (**A**) and CML patient 143-57-16 (**B**), P145$^{c\text{-}abl}$ from K562 (**C**), and P185$^{all\text{-}abl}$ from Ph^1-positive ALL patient 9312 (**D**) were autophosphorylated as in Fig. 1. Phosphorylated *abl* proteins were eluted from the gels, oxidized with performic acid, and digested with trypsin as described (*6*). Samples (800 to 2000 count/min) were separated by electrophoresis in one dimension on thin-layer cellulose plates, then by chromatography in the second dimension. The arrows mark the origins. Autoradiography was performed for 3 to 14 days.

Ph[1]-positive CML cells. Ph[1]-positive CML cells expressed the normal 6- and 7-kb c-*abl* transcripts and the 8-kb *bcr-abl* message (Fig. 4, lane 1). In contrast, Ph[1]-positive ALL cells from a patient previously shown to express P185$^{all\text{-}abl}$ did not show the 8-kb species, but expressed an abnormal 6.5-kb *abl*-related band (Fig. 4, lane 2). The 6.5-kb *abl* transcript was not seen in Ph[1]-negative ALL cells (Fig. 4, lane 3), which did express the normal 6.0- and 7.0-kb c-*abl* species. A 2-kb *bcr* complementary DNA probe representing a 5′ portion of the chimeric 8-kb *bcr-abl* mRNA (*4*) did not cross-hybridize detectably to the unusual 6.5-kb transcript in Ph[1]-positive ALL cells (*14*). Since the Ph[1]-positive ALL sample (Fig. 4) did not express the 8-kb *bcr-abl* message associated with Ph[1]-positive CML, we examined the structure of the *bcr* gene in several Ph[1]-positive ALL patients. As expected, genomic DNA digested with Bam HI and probed with genomic *bcr* probe revealed DNA rearrangement in one CML cell line and two CML patient samples (Fig. 5, lanes 1 to 3). All three Ph[1]-positive and two Ph[1]-negative ALL patients examined did not show detectable rearrangement in the *bcr* region (Fig. 5, lanes 4 to 8).

In both CML and ALL, altered *abl* protein expression correlates with the presence of a cytogenetically similar Ph[1] chromosome. The striking difference in the *abl* gene products expressed between the two diseases suggests that different molecular mechanisms of expression are involved that may relate to their distinctive clinical presentation. One Ph[1]-positive patient who expressed P210$^{bcr\text{-}abl}$ had initially presented with an ALL syndrome and later developed characteristic clinical features of CML (*14*). We have also demonstrated the presence of P210$^{bcr\text{-}abl}$ in Ph[1]-positive lymphoblastoid cells from CML patients (*9*). Thus, P185$^{all\text{-}abl}$ may be characteristic of Ph[1]-positive ALL and not of Ph[1]-positive lymphoid cells in general. The presence of P185$^{all\text{-}abl}$ in both adult (Fig. 1E) and childhood (Fig. 1, D, F, and G) Ph[1]-positive ALL suggests that *abl* expression in adult and pediatric Ph[1]-positive ALL may have a similar molecular basis.

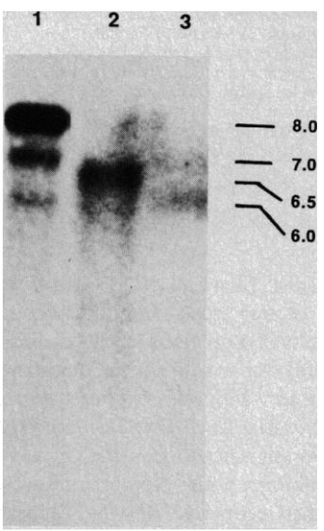

Fig. 4. RNA transfer blot analysis. Polyadenylated RNA (5 μg) from K562 (lane 1), and total RNA (20 μg) from Ph[1]-positive patient 9556 (lane 2) and Ph[1]-negative patient 9913 (lane 3) was separated by electrophoresis through a denaturing 1% agarose/formaldehyde gel and transferred to nitrocellulose. The filter was hybridized with a nick-translated complementary DNA probe from the conserved c-*abl* tyrosine kinase domain [probe D of Mes-Masson *et al.* (*4*)].

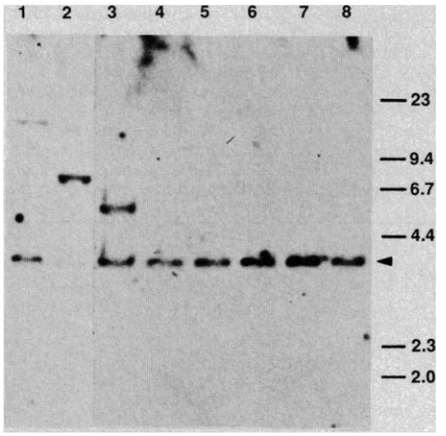

Fig. 5. Analysis of genomic DNA from Ph[1]-positive and -negative patients by a *bcr* genomic probe. High molecular weight DNA (10 μg) from Ph[1]-positive CML patients 133-68-56 (lane 1) and 143-57-16 (lane 3), Ph[1]-positive CML-derived cell line EM-2 (lane 2), Ph[1]-positive ALL patients 9378 (lane 4), 9453 (lane 5), and 045-14-02 (lane 6), and Ph[1]-negative ALL patients 9907 (lane 7) and 9913 (lane 8) was digested with Bam HI and fractionated on an 0.8% agarose gel. The DNA was blotted to nitrocellulose and hybridized with a nick-translated 1.2-kb genomic *bcr* restriction fragment that has been used by Groffen *et al.* (*2*) to detect *bcr* rearrangement in Ph[1]-positive CML patients. The arrow indicates the germline c-*bcr* band.

In Ph[1]-positive CML, the creation of the *bcr-abl* fusion gene is responsible for altered c-*abl* mRNA and protein expression (*3–5*). In Ph[1]-positive ALL, in situ hybridization and genomic DNA analysis of ALL cells and mouse-ALL hybrids (*11*) suggested that the translocation event may occur 5′ to the breakpoint cluster region of the *bcr* gene. While our data cannot distinguish whether the breakpoint occurs within or 5′ to the *bcr* gene, either mechanism could generate altered *abl* gene products. Because unique *abl* protein and RNA expression was found in one sample, it is possible that the 6.5-kb transcript encodes the P185$^{all\text{-}abl}$ protein. Either the formation of a fusion gene between c-*abl* and another undetermined gene, or unusual RNA splicing within the c-*abl* gene product may account for the expression of P185$^{all\text{-}abl}$.

Formation of the *bcr-abl* chimeric gene in CML results in elevated levels of an altered *abl* tyrosine kinase (*9*), and clearly affects the way in which the c-*abl* gene product interacts with kinase substrates in vitro. Quantitative and/or qualitative changes in *abl* expression may be important in CML pathogenesis. Our data show that a unique *abl* gene product, P185$^{all\text{-}abl}$, is expressed at high levels in Ph[1]-positive ALL. The autophosphorylation pattern of P185$^{all\text{-}abl}$ is more like that of P145$^{c\text{-}abl}$ than of P210$^{bcr\text{-}abl}$, which suggests that quantitative changes in *abl* kinase activity may be important in Ph[1]-positive ALL leukemogenesis.

Despite similar cytogenetic abnormalities, Ph[1]-positive CML and ALL represent different hematopoietic malignancies. In both leukemias, structural alterations in the c-*abl* protein result in distinctive molecular structures with elevated tyrosine kinase activity.

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13. Blood or bone marrow was drawn from patients after informed consent. The light density fraction was collected, washed, and cells were stored frozen in liquid N_2 with dimethyl sulfoxide as the cryopreservant. Karyotype examinations were performed either at St. Jude Children's Research Hospital or at the UCLA Cytogenetics Laboratory. All patients designated as being Ph[1]-positive expressed the standard t(9;22) (q34;q11). In some cases, multiple Ph[1] chromosomes were present and additional chromosomal abnormalities not involving chromosome 9 were evident. Unless noted otherwise, all patients expressed a karyotypically normal chromosome 9.

Pediatric patient samples obtained from St. Jude Children's Hospital included the following: patient 9907 (Ph^1-negative T-cell ALL), 9913 (Ph^1-negative common ALL), 9453 (Ph^1-positive pre–B cell ALL), 9312 (Ph^1-positive common ALL with a deletion of the short arm of chromosome 9), 9254 (Ph^1-positive common ALL), 9378 (Ph^1-positive pre–B cell ALL) and 9556 (Ph^1-positive common ALL). Patients obtained from UCLA included the following: 045-14-02 (adult Ph^1-positive, pre–B cell ALL), 146-03-37 (Ph^1-positive blast-crisis CML), 143-57-16 (Ph^1-positive blast-crisis CML), and 133-68-56 (Ph^1-positive accelerated CML).

14. S. Clark *et al.*, unpublished data.

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Technical Comments

Bacteria: Link or Sink?

Ducklow *et al.* (*1*) conclude from an experimental study of an enclosed water column at Loch Ewe, Scotland, that bacteria were not a link (that is, a food source) to higher order consumers in the planktonic food web. We strongly disagree that "the results have great importance for our understanding of the structure and function of marine planktonic ecosystems."

First, one cannot generalize from a single "link or sink" experiment. It will be necessary to carry out many such tracer studies in different parts of the sea representing a variety of trophic states, and to have a detailed characterization of the planktonic assemblage of each, before we can begin to discover the importance of the microbial loop in marine food webs. Ducklow *et al.* do not provide information about the components of the planktonic assemblage, that is, heterotrophic microorganisms, phytoplankton, and zooplankton, at the time of their experiment. In addition, the work carried out 9 years previously (*2*) that was cited in support of the statement that "The plankton community . . . was an assemblage of bacteria, protozoans . . . typical of the . . . Scottish sea lochs in mid- to late spring" in fact contained no data on bacterioplankton or protozoan numbers. Without information on the relative abundances and production rates of organisms of the microbial loop, that is, bacteria and protozoa, compared to those of the phytoplankton and metazooplankton assemblages, one cannot properly interpret the results of the experiment.

Second, the experimental study of Parsons *et al.* (*3*), briefly mentioned by Ducklow *et al.*, provided direct evidence that bacteria can be a link in marine food webs. Addition of small quantities of glucose (1 to 5 mg per liter) to enclosed water columns enhanced bacterial production, which in turn significantly increased the total abundance of benthic larvae and gelatinous zooplankton compared to a control treatment with predominantly phytoplankton production.

Third, the speculation of Ducklow *et al.* that production of cyanobacteria as well as of bacteria may not be utilized in marine food webs is not supported by the results of their study. Cyanobacteria in the sea have an average cell volume of 0.5 μm^3 (*4*), while the average cell volume of marine bacteria is 0.07 μm^3 (*5*). The larger cyanobacterial cells are likely to be grazed more effectively than are bacteria by marine pelagic ciliates (*6*), which are abundant in the sea and are a known food source for metazooplankton (*7*). Iturriaga and Mitchell (*4*) demonstrated with autoradiography that protozoa in the size range of 10 to 50 μm actively grazed $^{14}CO_2$-labeled cyanobacteria in surface waters of the oligotrophic North Pacific Ocean. They also showed that carnivorous metazoans incorporated the label, implying at least a two-step transfer of cyanobacterial carbon in the food web.

Finally, results from a similar bag experiment previously carried out in the same system, in which the fate of phytoplankton production was assessed by monitoring the distribution of ^{14}C in the water after a spike of radiolabeled bicarbonate was added (*8*), showed that, at the end of 10 days, the herbivorous zooplankton (68-μm and 250-μm size fractions) had incorporated only 1.2% of the ^{14}C activity present in the phytoplankton (*8*). Since this apparent trophic transfer from algae to zooplankton is of the same small magnitude as that reported by Ducklow *et al.* for the transfer of bacterial production to larger organisms, it is misleading to single out bacteria as a sink.

Ducklow *et al.* are correct in stating that further ecosystem-scale experiments should be done in order to address the question of bacteria as a link or sink for organic carbon in marine food webs. Their results, based on a one-season experiment carried out in a coastal ecosystem atypical of most of the world ocean, have not settled the matter.

EVELYN B. SHERR
BARRY F. SHERR
University of Georgia Marine Institute, Sapelo Island, GA 31327
LAWRENCE J. ALBRIGHT
Biological Sciences Department, Simon Fraser University, Burnaby, British Columbia V5A 156, Canada

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Response: Sherr *et al.* (*1*) criticize the design of our study, our interpretation of the results of a previous study (*2*) and of our own, and our conclusions. They imply that because an analogous experimental study of herbivory in a Loch Ewe bag (*3*) also failed to show significant transfer of carbon to zooplankton, both experiments were not performed properly. However, it is clearly shown in (*3*) that, during the earlier study, there was appreciable net primary production only on the three sunniest days. During the major part of the experiment nighttime respiration nearly balanced daytime carbon fixation, and it is thus not too surprising that the herbivores accumulated the label slowly. In contrast, in our experiment there was a rapid and sustained (4-day) accumula-