whether a pattern of heterogeneity existed for the panel of markers assessed. Furthermore, intratumoral heterogeneity and congruity with the primary tumor was evaluated for concordance.

Multiple polymorphic microsatellite markers were chosen for their informativity and frequency in melanoma tumors. In addition, many tumors were assessed to allow for sufficient number of LOH events to occur to avoid a conclusion of homogeneity based on retention alone or heterogeneity due to nonlinear random occurrences.⁵ To further determine whether results were consistent, intratumor heterogeneity was assessed. However, to avoid false monoclonality interpretation due to inadequate sampling from "patch size" clones, three separate regions were chosen which were widely spaced and randomly selected.⁶ Additionally, a large enough sample was microdissected from each specimen to ensure a sufficient number of cells and DNA quantitated for conformity. Finally, primary tumor blocks were assessed in a similar fashion to confirm the findings with concordant patterns of the genetic markers assessed. The fact that consistency was demonstrated through all three aspects of the investigation confirms the reliability of the methodology in this study and leaves little doubt that these results occurred randomly for this specific disease entity.

We agree that good laboratory practice under rigorous standard operating procedures must be strictly adhered to for any laboratory that is assessing LOH. Optimal sample conditions and repetitive assessments should be routine for accurately assessing allelic imbalances (AI). As the biology and relevance of these AI is still developing, optimal standardization has not been consistent in the literature. Assessment for clonality in tumor specimens using assays previously reported as the authors suggest does have limitations in interpretation. With regard to assessing molecular markers in relation to a tumor's natural history, all of the lesions were of the in-transit type, which is a unique model for this field of investigation. However, an inherent problem with any study evaluating patients' tumors is the inability to collect all specimens at identical time points in the disease progression spectrum. The accumulation of genetic alterations is a continuum for each individual tumor cell and thus one can never obtain sufficient number of specimens all at the same time point during neoplastic transformation and progression to make an absolute conclusion with certainty. We recognize the authors' concern regarding timing between test conversion and clonal expansion as well as lesion cell kinetics but this method provides the most clinically relevant approach for evaluating the unique pathology and biology of in-transit melanoma disease. In-transit recurrence is consistent with dormant tumor cell clones from the primary tumor trapped in intervening lymphatics and our findings provide a genetic association for this clinical experience.

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Interleukin-3 Receptors in Hodgkin's Disease

To the Editor-in-Chief:

Growing evidence suggests that deregulated apoptosis is a frequent occurrence in a variety of human malignancies.¹ The tumor cells in classical Hodgkin's disease (HD), historically named Hodgkin and Reed-Sternberg (HRS) cells, derive from germinal center B cells and often contain "crippling" somatic mutations within rearranged immunoglobulin (Ig) heavy chain genes.² Because such "crippling" mutations trigger apoptosis in germinal center B cells, their detection in HRS tumor cells indicate the presence of survival factors other than surface Ig.

In the February 2002 issue of *The American Journal of Pathology*, Aldinucci et al³ reported on the expression of interleukin-3 receptors (IL-3R) in HRS cells. As the authors pointed out correctly, it is quite surprising that IL-3R expression has not yet been investigated in the context of HD because of the ligand for this receptor, IL-3, also called multiclonal-stimulating factor, is probably one of the least restricted growth factors, exerting its effects on hemopoietic stem cells and progenitors of numerous lineages,⁴ including the lymphoid lineage, as shown by *in vitro* differentiation of IL-3-dependent B-cell precursors into mature B cells.⁵ In addition to describing IL-3R expression in HRS cells, Aldinucci et al³ examined a number of HD-derived cell lines to address the functionality of these receptors. Among the HD cell lines tested, IL-3R expression levels showed a remarkable variability. However, the increased growth rates of cultured HD cells on stimulation with IL-3 did not strictly reflect the differences in IL-3R expression. The fact that L1236 cells, which express low levels of IL-3R, had a stronger response than any other HD cell line to exogenous IL-3, should remind us to interpret data obtained from cultured HD cells with great caution. The relatively minor growth response of the

Figure 1. Surface expression of IL-3R in HRS cells. IL-3R surface staining (**A**) and Hoechst nuclear staining (**B**) of a nodular sclerosis HD-involved lymph node suspension. The **diagonal arrows** point to an IL-3R-positive HRS cell. Bar, 10 μ m. **C:** Copy numbers of IL-3R α chains on the surface of IL-3R-positive cord blood (CB) and HRS cells from one case of lymphocyte rich classical (LR) and three cases of nodular sclerosis HD (NS₁, NS₂, NS₃). Fluorescence intensities of labeled synthetic calibration beads with known numbers of coupled IgG_{2a} were used to calibrate receptor densities of 10 cells in each sample.

strongly IL-3R-positive HDLM2 cells, on the other hand, could simply reflect a partial IL-3 independency due to activating mutations in the receptor β chain.⁶

We tested HRS cells for expression of IL-3R because of the role of this receptor as a suppressor of apoptosis in hemopoietic progenitor cells⁷ and, secondly, because of the practical importance of IL-3R expression by tumor cells, since signaling through IL-3R also antagonizes cell death induced by chemical compounds used in cancer therapy.⁸ Using a previously described quantitative immunodetection assay,⁹ one mononuclear cell suspension obtained from cord blood (Figure 1C, CB) and four lymph node cell suspensions from one case of lymphocyte-rich classical (Figure 1C, LR) and three cases of nodular sclerosis HD (Figure 1C, NS₁, NS₂, NS₃) were surface-labeled with anti-IL-3R α chain-specific murine IgG_{2a} (7G3, Cambridge Bioscience, UK) and phycoerythrin (PE)-conjugated anti-mouse IgG (DAKO, Glostrup, Denmark). The levels of IL-3R on the surface of HRS cells were strikingly similar to the levels observed on cord blood progenitor cells (Figure 1C). In HRS cells, signaling through the IL-3R, if factor-dependent, could indeed be triggered by exogenous IL-3, since the reactive component in tumors of HD consists predominantly of T cells, the main source of IL-3.¹⁰ Since IL-3 also plays a role in the development of human dendritic cells,¹¹ signaling through the IL-3R could induce HRS cells to adopt their well-documented partial dendritic phenotype.

The study by Aldinucci et al³ is inspiring in that it provides evidence for a role of IL-3R in enhancing tumor

cell growth in HD. Future studies aiming at the elucidation of the functional state of IL-3R in primary HRS tumor cells will help clarify the role of this cytokine receptor in the pathogenesis of HD.

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Authors' Reply:

We read with interest the comments by Dr. Bosshart regarding our recent report on the expression of functional interleukin-3 receptors (IL-3Rs) on Hodgkin and Reed-Sternberg (H-RS) cells of Hodgkin's disease (HD).¹ In particular, in his letter, the author raises some issues regarding the consistency between the expression levels of IL-3Rs in H-RS cells and some results of the functional assays carried out by us to demonstrate the activity of IL-3Rs, at least in the model of HD-derived cell lines.

As shown in our original study, all of the HD-derived cell lines do express, although at significantly different levels, IL-3R α and β chain transcripts, as investigated by RT-PCR, as well as IL-3R α protein in Western blotting.¹ Overall these results are in keeping with the post-germinal center B cell derivation of classical HD, as formally proven by immunoglobulin gene rearrangement studies.² Flow cytometry analyses, by confirming this data, clearly

identify the L428 and HDLM2 cell lines as expressing the highest levels of IL-3R α , ie, comprised within the third logarithm (log) of fluorescence intensity, while the L1236 and KMH2 express an intensity for IL-3R α at least one log lower.¹ These results are in agreement with data recently published by Bosshart himself³ using a different methodological approach. Consistently, also in primary H-RS cells, staining for IL-3R α , although detected in all cases, showed a variable intensity at the cell membrane, in some instances associated with a cytoplasmic labeling.¹

The observation that the expression levels of IL-3Rs were not consistent with the effect of its engagement found by us in some functional assays seems to us only partly appropriate. As an example, HDLM2 cells, expressing high levels of IL-3Rs, are usually more sensitive, both in proliferative and anti-apoptotic assays, to exogenous IL-3 than KMH2 cells, which express IL-3Rs at intermediate intensity.¹ Moreover, L540 cells, expressing IL-3Rs at a very low level, were basically unaffected, in terms of proliferation increase, by IL-3 exposure.¹ However, the response to exogenous IL-3 of L1236 cells, at least by considering the proliferation increase, was stronger than expected by solely considering the levels of IL-3Rs.¹ This discrepancy could be explained, according to Dr. Bosshart, by the presence of activating point mutations in the receptor β -chain.⁴ Although this suggestion deserves to be investigated in detail, the observation that the greater responsiveness of L1236 cells to exogenous IL-3 was circumscribed to the effect on proliferation but not on the rescue of apoptosis, could indicate some simpler alternative hypotheses. In particular, the L1236 cell line, as opposed to the other HD-derived cell lines, have a slower growth rate with a significantly longer doubling time (in our hands, 96 to 120 hours) than the other cell lines, which have doubling times of about 48 hours (L-428, L-540 and KMH2) or about 72 hours (HDLM-2). Therefore, it is easier to document an increased proliferation by exogenous factors in cell lines characterized, as the L1236, by a low basal proliferative rate.

Another interesting comment by Dr. Bosshart regards the putative source of IL-3 to be found in T cells accumulating as part of the reactive component in HD-involved tissues. Although in our original work we do not provide evidence of IL-3 production by microenvironmental T cells,¹ some data regarding the possibility that H-RS cells could modulate the production of IL-3 by T cells themselves have been produced. In this regard, immunomagnetically purified activated T cells from nor-

mal donors, pre-activated on exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA, 10 ng/ml) and ionomycin (1 μ g/ml), were cultured (10:1) along with 1% paraformaldehyde-fixed, HD-derived cell lines. Supernatants were harvested after 3 days of culture and assessed by immunoassay (ELISA) for the release of IL-3. While IL-3 production of activated T cells in control medium (ie, without fixed HD cell lines) was 3.3 ± 0.1 ng/ml, all HD cell lines tested demonstrated an increase of the release of IL-3 by T cells, ranging from 7.15 ± 0.6 ng/ml (KMH2 cells), 6.6 ± 0.1 ng/ml (L1236 cells), 5.6 ± 0.5 ng/ml (HDLM-2 cells), 5.3 ± 0.4 ng/ml (L540 cells), and 4.5 ± 0.4 ng/ml (L428 cells). These results suggest an active role of IL-3R-expressing H-RS cells in the production of IL-3 itself by T cells.

In conclusion, given the peculiar features of HD, a unique pathological condition in which a minority, usually less than 1 to 2%, of clonal neoplastic cells are embedded in a heterogeneous background of non-malignant bystander cells,⁵ *bona fide* HD-derived cell lines remain, to date, invaluable tools for investigating the cytokine-dependent interactions and the cytokine-receptors repertoire of H-RS cells. Nevertheless, notions derived from these studies should be confirmed and validated by additional investigations involving primary H-RS or carried out cells in the context of fresh HD-involved tissues.

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