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Immunology

Phylogenetically conserved antigen on nerve cells and lymphocytes resembles myelin-associated glycoprotein

(neuron–lymphocyte shared antigen/HNK-1 antibody)

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ABSTRACT **The HNK-1 (Leu 7) and NC-1 monoclonal antibodies, raised against a human T-cell line and against nerve cells of quail embryos, respectively, have been shown to bind to a shared epitope present on the surface of human large granular lymphocytes and on nerve cells in species ranging from amphibians to humans. We demonstrate that a related antigen is also expressed on the lymphocyte surface in the avian central lymphoid organs, thymus and bursa, and in the spleen during embryonic and adult life. The expression of the HNK-1/NC-1-reactive determinant differs remarkably in the bursal and thymic compartments, antigen expression being stabilized at a high level early in development of the bursa, whereas its expression fluctuates in the thymus. The material immunoprecipitated from bursal and thymic lymphocytes by the HNK-1/NC-1 antibodies exhibits the same relative molecular mass as myelin-associated glycoprotein, which is one of the molecules recognized by these antibodies in the nervous system. Together with the observation that an antiserum reactive with the protein part of chicken myelin-associated glycoprotein detects similar material in membrane extracts of HNK-1/NC-1-positive thymocytes, this suggests that a molecule sharing structural analogies with this nerve cell component is expressed on cells of the immune system.**

Cell differentiation antigens may be shared by neural and hemopoietic cells. The prototype of such shared antigens is the murine θ or Thy-1 molecule, which is expressed on the surface of both the thymus-derived T cells and nerve cells (1). More recently, the monoclonal antibody named HNK-1 (Leu 7), raised against a human thymoma-cell line, was shown to identify granular lymphocytes with natural killer (NK) and antibody-dependent killer (K) capabilities (2) and to bind also to myelin sheath components in the central and peripheral nervous systems of humans and rodents (3–5). In nerve cells, HNK-1 binds to myelin-associated glycoprotein (MAG) (6) at a carbohydrate-dependent epitope (4, 7).

HNK-1 has been found to have the same cellular specificity in humans, rodents, birds, and amphibians as NC-1 (8), an antibody raised against ciliary ganglion cells of quail embryos. The NC-1 antibody recognizes a neuroectodermal antigen carried by migrating neural crest cells and by cells developing later in the central and peripheral nervous system (9). Similar reactivities have been observed in reptilian species (unpublished observation). It thus appears that the HNK-1 and NC-1 antibodies, raised against hemopoietic and neural cells from mammalian and avian species, respectively, recognize a highly conserved antigenic moiety.

We now report that the HNK-1/NC-1 epitope is present on avian lymphocytes of both B and T lineages. Examination of the expression of this epitope during ontogeny revealed distinctive developmental patterns for cells of the two lymphoid pathways. We found that the HNK-1/NC-1-reactive antigen on avian lymphocytes is similar to MAG in relative molecular mass (M_r), and the resemblance to MAG of the HNK-1/NC-1-defined antigen on avian lymphocytes is further supported by reactivity of avian lymphocytes with a heterologous antiserum against the protein moiety of MAG.

MATERIALS AND METHODS

Animals. Quails (*Coturnix coturnix japonica*) and chickens (*Gallus gallus*), embryonic and newly hatched, were purchased from local breeders. Fertile eggs were incubated at 38°C in a humid atmosphere until the desired developmental stages were reached.

Construction of Quail–Chick Chimeric Thymuses. Chimerism of the lymphocyte population in the embryonic thymus was achieved by grafting the thymic rudiment from a quail embryo at 7 days of incubation into the somatopleure of a 3.5-day chicken embryo. Under these conditions, the first generation of thymic lymphocytes are derived from quail hemopoietic stem cells (HC) that invade the rudiment from the 5th to 6th days. The quail thymocytes are then progressively replaced by lymphocytes of chicken host origin, the precursors of which seed the grafted thymus between 11 and 12 days of total age (10). Various degrees of thymocyte chimerism were obtained by maintaining the quail embryonic rudiment from 5 to 13 days in the chicken host.

Cell Suspensions. Lymphoid organs were mechanically disrupted in Dulbecco's phosphate-buffered saline supplemented with 2% heat-inactivated newborn calf serum (Gibco; Hoofddorp, The Netherlands). Debris was discarded after sedimentation, dead cells were removed by centrifugation through a Ficoll/Hypaque gradient, and the viable cells were washed in the buffer.

Tissue Sections. Lymphoid organs were removed, fixed for 4 hr with 4% paraformaldehyde in 0.05 M sodium phosphate buffer (pH 7.4), rinsed in the buffer, gradually dehydrated in alcohol, and impregnated and embedded in polyethylene glycol 1500 (Serva, Heidelberg) (11). Free-floating 7-μm sections were further processed for immunofluorescent staining as described below.

Antibodies. HNK-1, NC-1, and MB1 monoclonal antibodies were prepared (2, 9, 12), purified, and directly labeled

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Abbreviations: MAG, myelin-associated glycoprotein; HC, hemopoietic stem cell; E*n*, embryonic age in *n* days; FITC, fluorescein isothiocyanate.

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with fluorescein isothiocyanate (FITC) in our laboratories. The anti-MAG serum was prepared as follows. Myelin was isolated from whole brains of 2-month-old chickens by the method of Norton and Poduslo (13). Myelin-associated proteins were extracted from pure myelin by precipitation with lithium diiodosalicylate, as described by Quarles and Pasnak (14). Rabbits received three intradermal footpad injections of 100 μg of lithium diiodosalicylate extract emulsified in Freund's adjuvant at 2- to 4-week intervals. Immune serum was passed over an affinity column prepared by coupling a lithium diiodosalicylate extract deglycosylated with trifluoromethanesulfonic acid (15) to CNBr-activated Sepharose CL-4B (Sigma), and bound antibodies were eluted at acidic pH.

Immunofluorescence. Cell suspensions and tissue sections were processed for immunofluorescent staining as previously described (16, 17). Both direct (with HNK-1-, MB- and NC1-FITC reagents) and indirect immunofluorescence stains were used. In the latter case, reactions with monoclonal antibodies were revealed by using purified goat antibodies to mouse immunoglobulin (Ig) determinants; the goat antibodies (Southern Biotechnology Associates; Nordic, Tilburg, The Netherlands) were labeled with either FITC or tetramethylrhodamine isothiocyanate. Rabbit anti-MAG serum was indirectly labeled with an FITC-conjugated goat anti-rabbit reagent (Nordic). Two-color immunofluorescence was performed by sequential staining of tissue sections with monoclonal antibodies labeled either directly or indirectly.

Fluorescence-Activated Cell Sorter Analysis. Suspensions of cells labeled with either NC-1-FITC or HNK-1-FITC were analyzed by automated flow cytometry on a FACS IV (Becton Dickinson) equipped with a 5-W argon laser.

Molecular Weight Estimation of HNK-1/NC-1-Reactive Antigens. Viable thymus and bursa cells were radioiodinated with $Na^{125}I$ by the lactoperoxidase technique (18), then lysed with 0.5% Nonidet P-40. A solid-phase immunoadsorption technique was used to isolate soluble antigens in the cell lysates (19). In this procedure, each well in a 96-well microtiter plate (Cook, Alexandria, VA) was coated with 200 μl of goat anti-mouse Ig (100 μg/ml) by overnight incubation at 4°C, and then with 200 μl of a hybridoma supernatant containing the HNK-1 or NC-1 antibody. Aliquots of the antigens dissociated from the immunocomplexes in the presence or absence of 2-mercaptoethanol were compared with protein standards by $NaDodSO_4$/PAGE analysis using 7.5% gels in the Laemmli system (20). Alternatively, unlabeled thymocyte membrane proteins were separated by $NaDodSO_4$/PAGE, transferred electrophoretically to nitrocellulose, and treated with antibodies by using the method of Towbin *et al.* (21) with modification (12). Nitrocellulose tracks were incubated successively in the following reagent solutions: rabbit serum anti-chicken MAG 1:50, goat Ig anti-rabbit Ig coupled to biotin (Amersham) 1:50, and streptavidin–biotin-peroxidase complex (Amersham) 1:200. Reactions were visualized with 3-amino-9-ethylcarbazole (Sigma) in the presence of H_2O_2.

RESULTS

HNK-1/NC-1 Antibody Reactivity in the Quail Thymus and Bursa. Tissue sections of quail thymuses from 14-day embryos were examined for reactivity with the HNK-1 and NC-1 antibodies by using an indirect immunofluorescence assay. Both reagents were found to stain brightly a subpopulation of the cortical lymphocytes (Fig. 1*A*). Labeled thymocytes were concentrated in the outer cortex and were almost totally absent from the medulla. In the latter area, occasional cells of nonlymphoid morphology were stained; these exhibited an irregular shape and frequent cell processes.

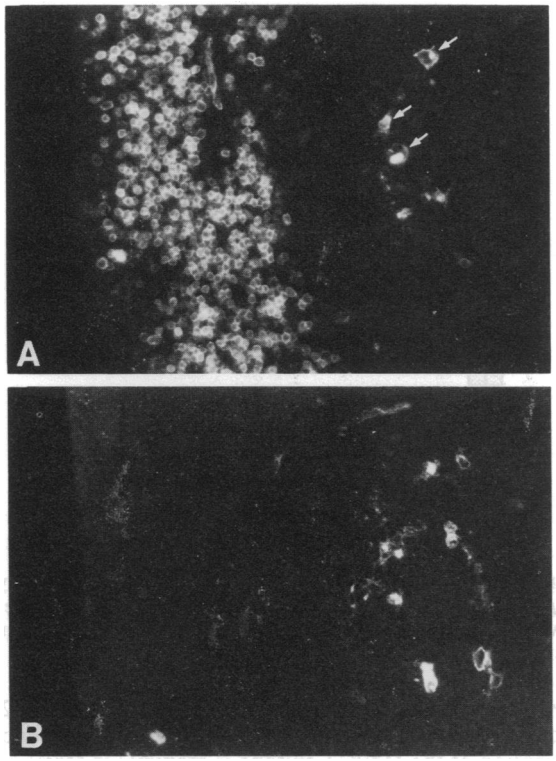

FIG. 1. Transient expression of NC-1-defined antigen in the quail embryo thymus. (*A*) Transverse section of a 14-day thymus. Labeled cells are confined to the cortical area. In the medullary region, only scattered nonlymphoid cells are stained (arrows). (*B*) A 15-day thymus, devoid of NC-1-positive lymphocytes. Nonlymphoid cells that are labeled can still be seen in the medulla. (×140.)

When sections of the quail bursa were stained under the same conditions, the vast majority of the cells in lymphoid follicles were found to be positive; the fluorescence intensity was highly variable and generally less than that seen for thymus sections (not shown).

Ontogeny of HNK-1/NC-1 Defined Antigen in the Avian Thymus. The ontogeny of HNK-1/NC-1 antigen expression was examined by using both tissue sections and cell suspensions of quail thymuses from day 6 of the embryonic age (E6) until 2 months after hatching. No antigen expression could be detected before E12. From E12 to 8 days after hatching, daily cell counts of HNK-1-positive cells were performed on thymocyte samples from individual quails (number ≤15 per day). From E12 onward, immunoreactive lymphocytes were always found in a certain proportion of the individuals examined. Individuals with fewer than 1% $HNK\text{-}1^+$ lymphocytes were found at all ages (Fig. 1*B*), whereas other individuals of the same age had up to 60% $HNK\text{-}1^+$ thymocytes.

Analysis of the data suggested two distinctive periods of HNK-1/NC-1 epitope expression, one period from E12 to E16 and the other from 1 to 8 days after hatching. The distribution of the values for the two age groups is shown in Fig. 2. A comparison of the proportions of positive and negative cases by χ^2 analysis revealed a statistically significant difference ($\chi^2 = 9.94$ for 1 df, $P < 0.05$) for the two age groups. However, the positive values were extremely variable and there is no apparent difference in the positive values between the two groups, the means being 21.9% $HNK\text{-}1^+$ cells before hatching and 25.3% after hatching.

In conclusion, three periods of HNK-1/NC-1 immunoreactivity can be distinguished in thymocyte ontogeny. The first is between E6, when HC colonization of the thymus is completed, and E12, when the first HNK-1-immunoreactive

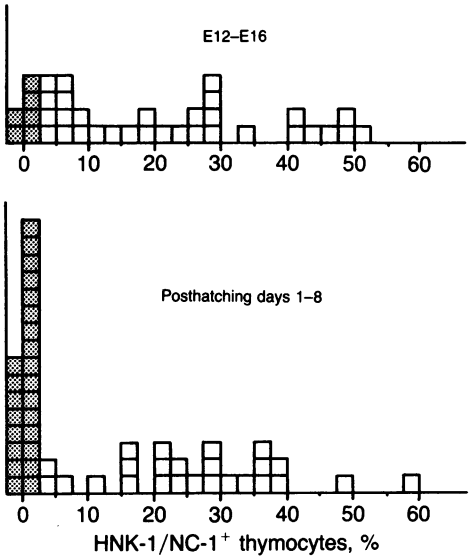

FIG. 2. Percentages of HNK-1/NC-1$^+$ thymocytes in embryonic (*Upper*) and newly hatched (*Lower*) quails. Each square represents the value observed in the thymus of a single animal. Stippled squares illustrate cases considered as null (<1% positive cells).

T lymphocytes appear. The second period, between E12 and hatching, corresponds to the time when the frequency of expression of the HNK-1 epitope is maximal in terms of the relatively low frequency (13.5%) of negative cases. During the third period, corresponding to the first week after hatching, the differences in HNK-1/NC-1 epitope expression by different individuals become more striking, the percentage of negative cases reaching 47%.

Expression of the HNK-1-defined antigen by thymocytes was also investigated in chickens as a function of their developmental age, and expression was found to occur less frequently than in the quail. Thymocyte immunoreactivity was observed only in a minority of chicken embryos at E14 and at 4 days after hatching.

Analysis of HNK-1 Epitope Expression by Thymocytes in Quail/Chicken Chimeras. Thymus colonization proceeds by waves, the periodicity of which has been determined by analysis of HC colonization and subsequent lymphopoiesis in chicken/quail chimeric thymuses (10). Statistical analysis of the data on HNK-1 antigen expression by thymocytes did not reveal a clear parallel to these waves of thymopoiesis. Therefore, to examine this relationship, we used embryonic constructs of quail/chicken chimeric thymuses and the quail-specific marker of HC identified by the MB1 monoclonal antibody (12). In such chimeric organs, quail thymocytes derived from the first wave of HC colonization between 5 and 6 days of incubation in the quail-thymus donor are progressively replaced by lymphocytes of chicken-host origin. Double-labeling experiments performed on sections of these organs, with both HNK-1 antibody and the quail-specific MB1 antibody, revealed donor-quail origin of the HNK-1/NC-1$^+$ population on E13 (i.e., 7 days at the age of grafting plus 6 days in the host). At this stage the immunoreactive population is thus derived from the first wave of HC colonization. When observed after a total age of 15 days for the grafted thymus, the sections also revealed immunoreactivity in the external areas of the cortex occupied by chicken lymphoid cells derived from the second wave of HC (10). The data indicate a developmental delay of approximately 6–7 days in the HNK-1-defined antigen expression by thymocyte progeny of colonizing HC.

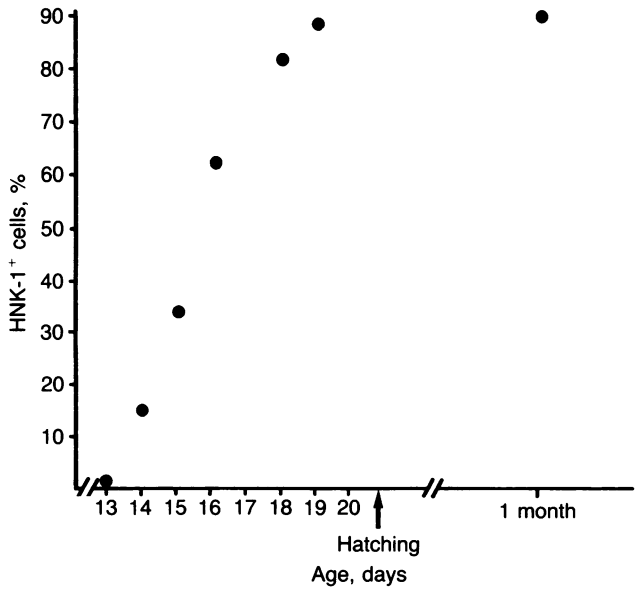

FIG. 3. Ontogeny of HNK-1$^+$ cells in the chicken bursa of Fabricius. Each point represents the value obtained for a pool of bursal cells from five or more embryos.

Ontogeny of HNK-1/NC-1-Reactive Cells in the Avian Bursa of Fabricius. Bursal cell suspensions were prepared as pooled samples from chicken embryos, and automated flow immunocytometry was used to examine HNK-1 antigen expression from E1 onward. HNK-1 antibody-reactive cells appeared in this organ on day 13 (Fig. 3), when approximately 2% of the bursal cells in suspension expressed this surface antigen. The percentage of positive cells in the bursa then increased progressively, reaching values of 90% or more by day 19. This high level of HNK-1 reactivity of chicken bursal lymphocytes persisted until involution of the lymphopoietic organ when the birds reached sexual maturity.

Similar expression of the HNK-1/NC-1 epitope was observed for quail bursal lymphocytes, but the ontogeny of antigen expression by quail bursacytes was not examined in detail.

HNK-1 Reactivity in the Avian Spleen. Investigation of HNK-1 reactivity in the spleens of embryonic and newly hatched quail by fluorescent labeling of tissue sections suggested transient expression of the defined antigen by cells in this peripheral lymphoid tissue. In addition to the expected reactivity with nerve cells, which was observed at all developmental stages, rare positive splenic leukocytes were observed from E15 to the fifth day after hatching. In all cases, labeled cells were located in the perivascular regions of the white pulp, the brightest cells being located in close proximity to the vessel lumen. From 5 to 15 days after hatching, the number of positive cells in perivascular areas of splenic sections was found to be highly variable.

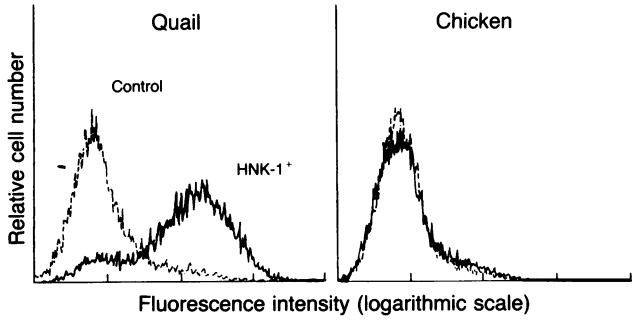

FIG. 4. Cytofluorographs of quail or chicken splenocytes surface-stained with HNK-1 followed by FITC-conjugated goat anti-mouse Ig. An unrelated monoclonal antibody was used as control.

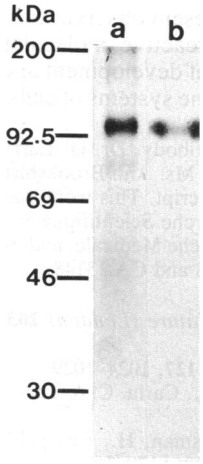

FIG. 5. NaDodSO$_4$/PAGE analysis of HNK-1 binding material on the lymphocyte surface. HNK-1 antibody was used in a solid-phase assay to immunoprecipitate target antigens from ^{125}I-labeled quail thymocyte (lane a) and bursacyte (lane b) cell membrane extracts. Reduced immunoprecipitates were run on a 7.5% acrylamide gel.

A remarkable difference was observed when suspensions of spleen cells from quails and chickens were examined for cell-surface reactivity with the HNK-1 antibody. A mean (±SEM) of 62% ± 3% HNK-1$^+$ cells was found in the spleens of quails 3–8 weeks of age, whereas few HNK-1$^+$ cells were detectable in the spleens of chickens at any age (Fig. 4).

Characterization of HNK-1- and NC-1-Reactive Molecules on Thymic and Bursal Lymphocytes. The HNK-1 and NC-1 antibodies were used to identify ^{125}I-labeled surface antigens on quail thymocytes and on quail and chicken bursa cells. Prior to iodination, immunofluorescence staining was performed to confirm antibody reactivity on quail thymocytes in the test sample. The two antibodies were found to identify the same bands separated by electrophoresis on NaDodSO$_4$/polyacrylamide gels. Under both reducing and nonreducing conditions, a major band with a relative molecular mass of approximately 110,000 was precipitated from thymic and bursal cells (Fig. 5). A prominent band of M_r 80,000 was also noted in some experiments (see lane a, Fig. 5). In addition, weaker bands of M_r 130,000 and 180,000 were apparent in thymocyte preparations but not in bursal cell preparations.

These results and those of previous biochemical analyses of human lymphocytes (22) indicate similarity between neural cell MAG and the HNK-1-reactive molecules on lymphocytes. To explore this possibility further, we used an antiserum that was raised against purified chicken myelin proteins and affinity-cleared of anti-carbohydrate activity. This reagent, tested by immunoblotting on a chicken myelin lithium diiodosalicylate extract, reacts as expected with several bands of M_r 56,000, 63,000, 80,000, 145,000–150,000, and mainly with a smear ranging from 95,000 to 116,000. The latter is also strongly HNK-1 positive and represents the MAG. However, this antiserum clearly does not recognize the HNK-1-defined carbohydrate epitope, since it is totally devoid of immune reactivity towards avian neural crest cells, known to be HNK-1$^+$ (8) (not shown). This anti-MAG reagent was used to examine blots of detergent-extracted membrane proteins from either HNK-1$^+$ or HNK-1$^-$ quail thymocytes. No reactivity was observed with extracts of HNK-1$^-$ lymphocytes. In contrast, in the membrane extract of a thymocyte population that included about 50% HNK-1$^+$ cells, two bands were stained, the apparent relative molecular masses of which were calculated to be 80,000 and 120,000 (Fig. 6).

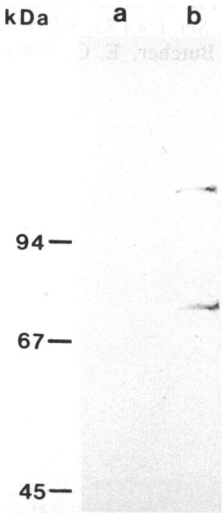

FIG. 6. Binding of a rabbit antiserum that recognizes the protein part of myelin-associated glycoprotein to quail thymocyte membrane antigens. Detergent-extracted membrane proteins from HNK-1$^-$ (lane a) and HNK-1$^+$ (lane b) thymocytes were run on a 7.5% acrylamide gel and blotted to nitrocellulose. Both lanes were treated with the antiserum and the reaction was visualized by the streptavidin/biotin/peroxidase/aminoethylcarbazole technique.

DISCUSSION

In the present experiments, we demonstrate expression of a HNK-1/NC-1-defined epitope on lymphocytes in the avian thymus, bursa, and spleen. This antigen thus appears to be phylogenetically conserved in birds and mammals not only in nervous tissues (3) but also on lymphocytes, large granular lymphocytes having been shown to express it in humans (2). By the use of both HNK-1 and NC-1 monoclonal antibodies in ontogenetic and immunochemical studies, we show that these two reagents react with the same molecules on the same subpopulations of avian lymphocytes.

For cells of the B lineage, a straightforward pattern of stage-specific expression of HNK-1 antigen was observed. On E12, when most of the HC precursors have entered the chicken bursa (23), HNK-1$^+$ cells could not be identified in bursal cell suspensions. HNK-1$^+$ cells appeared in the bursa on the following day and quickly filled the bursal follicular compartment. Essentially all of the mechanically dispersible cells expressed the HNK-1$^+$/IgM$^+$ phenotype by E18, 3 days before hatching. This high level of expression was found to be maintained throughout the adolescent period of bursal lymphopoiesis. In contrast, cells expressing the HNK-1/NC-1 epitope were rarely seen in blood and in the spleen. Thus the HNK-1/NC-1 epitope is an excellent stage-specific marker for B-lineage cells in the chicken, being absent on their hemopoietic precursors and present only during the intrabursal phase of B-cell differentiation.

The thymocyte pattern of HNK-1 immunoreactivity was found to be relatively complex. HNK-1$^+$ cells were first detectable in the thymus of 12-day quail embryos, 6 days after the initial HC influx. Beyond this developmental age, the percentage of reactive cells within the thymus was found to be highly variable from one bird to another, ranging from <1% to 60% positive thymocytes. The HNK-1$^+$ lymphocytes were confined to the cortical area of the thymus, the relatively mature thymocytes in the cortex being entirely negative for this antigen. A periodicity to the fluctuations in mean percentage of labeled thymocytes could not be distinguished over the course of thymus development. Specifically, no clear correlation could be observed between HNK-1 expression and the previously described cyclic generation of thymic lymphocytes during embryonic and postnatal life, although the number of individuals lacking detectable (<1%) HNK-1/NC-1$^+$ thymocytes was significantly lower in the late embryonic period than in the first week after hatching (13.5% and 47% negative individuals, respectively). The enormous variation in the frequency with which individual animals, of essentially the same developmental age, express this monomorphic determinant on their cortical thymocytes is intriguing. While the basis for this variability is presently unknown, the observations suggest a labile mechanism of inducibility of cortical thymocytes to express the HNK-1/NC-1 epitope.

Using chimeric thymuses, quail into chicken embryo hosts, to distinguish successive generations of extrinsic HC-derived

lymphocytes, we could show a delayed expression of the HNK-1/NC-1 epitope by the thymocyte progeny of the first wave of colonizing HC that invade the thymus rudiment between 5 and 6 days of development. Thymocytes deriving from the second HC influx, which occurs on the 11th day of incubation in the quail thymus (10), begin to express the HNK-1/NC-1 epitope from 15 days of thymic age in the chimeric thymuses. This indicates that the HNK-1/NC-1 epitope appears on the T-lineage cells after a 4- to 6-day delay following homing of their HC progenitors.

According to Kruse *et al.* (7) the neural cell molecules L1 and the neural cell adhesion molecule (N-CAM) share a carbohydrate epitope that is recognized by the monoclonal antibody HNK-1. However, no immunoreactivity has been reported on avian lymphocytes with antibody against N-CAM. On the other hand, reactivity of the HNK-1 antibody with MAG has been well documented (4, 6, 7). The major HNK-1-reactive molecule identified on avian thymocytes and bursacytes by immunoprecipitation of iodinated surface antigens was found to have a relative molecular mass of 110,000. Several additional minor components were observed in thymocyte preparations, the most prominent of which may resemble d-MAG, a well-known degradation product of MAG. HNK-1-reactive molecules of similar sizes are thus expressed by avian thymocytes and bursacytes, avian and mammalian nerve cells (7, 8), and human granular lymphocytes (22).

The issue of the identity of the M_r 110,000 molecules detected by HNK-1 antibody in neural and lymphoid cell types is an interesting one, particularly with regard to whether or not the polypeptide backbone carrying the recognized carbohydrate epitope is the same for all of these cell types. Noteworthy in this regard, monoclonal antibodies binding to polypeptide epitopes of the MAG glycoprotein lack immunoreactivity with human mononuclear cells reactive with HNK-1, whereas monoclonal antibodies directed against the sugar moiety of MAG react with the HNK-1^+ granular lymphocytes (24). However, we observed that an antiserum reacting with the peptidic moiety of MAG, but that does not detect its HNK-1-defined carbohydrate antigenic determinant, still binds two membrane components from HNK-1^+ thymocytes. In addition, these molecules are closely related in molecular mass to the main HNK-1-reactive thymocyte antigen. These findings thus suggest that HNK-1/NC-1 reactivity on lymphocytes reflects the actual expression by blood cells of MAG-related proteins, rather than a fortuitous sugar-linked crossreactivity between distinct components.

Among the candidate molecules responsible for governing lymphocyte migratory behavior, the best defined are those that in mice mediate the adherence of lymphocytes to the cuboidal endothelium of the venules in lymph nodes and Peyer's patches (25). These "homing" molecules are thought to be important in directing the traffic of specialized subpopulations of lymphocytes. In view of the present observations, we suggest that related HNK-1/NC-1-reactive molecules may play a role in governing the patterns of development and distribution of both the nervous and immune systems of cells.

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