PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 84–88 (1983)

Developmental Expression of Chicken Fetal Antigen on Peritoneal Macrophage: Implications for Hematopoietic Differentiation (41531)

R. R. DIETERT,[1] M. A. QURESHI, AND K. A. TREMBICKI

Department of Poultry and Avian Sciences, and Institute for Comparative and Environmental Toxicology, Rice Hall, Cornell University, Ithaca, New York 14853

Abstract. Chicken fetal antigen (CFA) was demonstrated by both complement-mediated microcytotoxicity and immunofluorescence to be present on the cell surface of activated peritoneal macrophage. While CFA-positive macrophage were observed in young birds, the antigen was absent from adult macrophage. A comparison of cell surface CFA on macrophage, lymphocytes, and erythrocytes indicated that unique subsets of CFA determinants are characteristic for each differentiation lineage. As a result, CFA can serve as a cell surface marker for differentiation-specific gene expression. Since CFA determinants are known to be at least partly defined by carbohydrates, it is suggested that hematopoietic heterogeneity for CFA may result from lineage-specific carbohydrate microheterogeneity.

Chicken fetal antigen (CFA) is a membrane-bound oncodevelopmental glycoprotein found on avian hematopoietic cells (1). CFA and an analogous erythrocyte antigen have been shown to be involved with erythroid differentiation (2, 3). Recently, CFA was also linked to lymphoid differentiation and development (4). Lymphocyte subsets within both B- and T-cell differentiation lineages appear to exhibit differential CFA expression. In addition, lymphocytes from secondary lymphoid organs are developmentally restricted for CFA expression (4, 5).

CFA activity has been shown to reside on a 48,000-dalton glycoprotein by specific immunoprecipitation from erythrocytes and lymphocytes (6). However, the antigen is known to be extremely complex, being composed of at least 13 antigenic determinants (7). At least one of these determinants is defined by a single carbohydrate residue (8, 9). As a result, it has been suggested that specific glycosyltransferases and/or glycosidases regulate the presence of individual cell surface CFA determinants (8).

While lymphocytes possess CFA, they share only a subset of the total antigenic determinants found on erythrocytes; these determinants have been termed lymphocyte-associated CFA (LA–CFA), as opposed to those CFA determinants restricted to erythrocytes which are referred to as erythrocyte-specific CFA (ES–CFA). Based on the finding that CFA may be used to study immune cell differentiation relative to other hematopoietic cells, we have expanded our analysis to encompass the reticuloendothelial differentiation lineage. Specifically, this paper describes the detection and developmental restriction of CFA on activated peritoneal macrophage. In addition, specific CFA expression of these cells is compared to that established for lymphoid and erythroid differentiation lineages.

Materials and Methods. Cornell K strain female White Leghorn chickens were the source of all cells used in this study (10). LSCC-CU10 (TLT-1) is a nonadherent avian leukosis lymphoblastoid cell line donated by Dr. K. A. Schat, Cornell University. This cell line was previously shown to shed avian leukosis virus but to lack B- or T-cell markers (11). As a result, the exact differentiation status of this cell line has yet to be established. LSCC-CU10 is positive for CFA (5) and was maintained in 42.4% Leibovitz's L15 Medium (Gibco), 42.4% McCoy's 5A Medium (Gibco), 8.5% fetal calf serum (Gibco), 6.8% chicken serum (Gibco) with 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml Fungizone at 37°, 5% CO_2 throughout the period of this study. Rabbit anti-CFA (R-anti-CFA), rabbit anti-CFA adsorbed with 6-week bursacytes, and rabbit anti-CFA inactivated by adsorption with 1-day chicken erythrocytes were all prepared as previously described, re-

[1] To whom all correspondence should be addressed.

0037-9727/83/010084-05$01.50/0

spectively (4, 12). Rabbit anti-CFA adsorbed with LSCC-CU10 was obtained by exhaustively adsorbing 500 μl of R-anti-CFA with 10^9 LSCC-CU10 cells at room temperature for 4 hr. The completeness of all adsorptions was determined by testing the reactivity of the adsorbed antisera against the adsorbing cell populations. All assays were conducted in triplicate using pooled cell samples from 4 to 10 chickens of identical age. Isolation of bursacytes and erythrocytes and subsequent complement-mediated microcytotoxicity assays on these cells and LSCC-CU10 cells were performed as previously described (4, 9).

Preliminary experiments indicated that stimulation was required for isolation of peritoneal macrophage from chickens. For production of activated chicken macrophage, a method was employed similar to one reported for the bovine system (13). In initial trials, this procedure was found to produce good yields of adherent cells containing a high percentage of activated macrophage. Peritoneal injections of 3% starch (Staley) in sterile 0.75% saline at a dosage of 1 ml/100 g body weight were given on each of 7 consecutive days. Following 1 day rest, peritoneal exudate cells were harvested on the ninth day by flushing the peritoneal cavity with 50 ml of sterile heparin (0.5 U/ml) in saline. Cells were collected in siliconized tubes, rinsed three times with cold RPMI 1640 + 100 U/ml penicillin + 50 μg/ml streptomycin. Pooled cells were plated on microhistocompatibility typing trays (Falcon) at a concentration required to produce approximately 2000 adherent cells per well. Following a 1-hr incubation at 37°, nonadherent cells were removed by repeated flushing of each well with RPMI 1640 + antibiotics. One microliter of growth medium was added to the adherent cells in each well and 10 μl of mineral oil was layered on top.

For CFA determination, complement-mediated microcytotoxicity using 1 μl of antiserum and 5 μl of guinea pig complement (Gibco) was performed as previously described for lymphocytes (4). Antibody, complement, and medium controls were run on each sample and specificity of the assay was demonstrated using R-anti-CFA totally inactivated by adsorption with 1-day chick erythrocytes (4). Maximum CFA-specific microcytotoxicity was used to determine the incidence of CFA-positive macrophage.

As a control for nonspecific phagocytosis during the assay, replicate plates of certain adherent cells were preincubated in 2 μ*M* colchicine (Calbiochem) in RPMI 1640 + antibiotics for 30 min at 37° prior to CFA analysis (13). These plates served as controls for nonspecific uptake of immunoglobulin or trypan blue stain under the assay conditions. Replicate adherent cell isolates on cover slips were used to determine the percentage of macrophage in the test cell population. Methanol-fixed slide preparations were stained with May–Grüwald–Giemsa (14). Morphological analysis of 500 adherent cells from each sample was performed (15).

For the immunofluorescence assay, a pool of 2×10^6 peritoneal exudate cells from four birds was rinsed in RPMI 1640, placed in 0.1 ml RPMI 1640, and reacted with 0.1 ml of R-anti-CFA (1/8) at 4° for 30 min. Following three rinses with cold RPMI 1640, the cells were further reacted with 0.1 ml goat anti-rabbit Ig labeled with FITC (1/8) (Cappel) for 30 min at 4°. After rinsing, cells were resuspended in 9:1 glycerol:0.1 *M* PBS (pH 7.2), placed on a slide, and the percentage fluorescence of 1000 cells was determined on an Olympus Vanox microscope by two independent observers.

Results. Both complement-mediated microcytotoxicity and immunofluorescence assays demonstrated the presence of CFA on peritoneal exudate cells from young chickens. Table I illustrates the percentage CFA-specific microcytotoxicity directed against the adherent population of peritoneal cells. Specificity of CFA detection was demonstrated in two ways. Inhibition of macrophage phagocytosis by colchicine treatment did not alter the reactivity of R-anti-CFA with these cells (Table I). Therefore, observed cytotoxicity was not due to nonspecific phagocytosis of either antibody or trypan blue stain. In addition, adsorption of R-anti-CFA with 1-day chicken erythrocytes totally abolished the reactivity of the antiserum against adherent cells (primarily activated macrophage) from 6-week-old chickens (Table II). Therefore, antibody-dependent complement-mediated microcytotoxicity of these cells was specific for CFA. In addition, analysis by immunofluorescence of peritoneal exudate cell samples from 6-week-old chickens indicated that 80.9% of the cells (adherent and nonadherent) were positive for

TABLE I. CFA-SPECIFIC MICROCYTOTOXICITY OF DEVELOPING ACTIVATED MACROPHAGE

Age of donor (weeks)	Percentage CFA-specific microcytotoxicity		Percentage macrophage in adherent cell preparations
	Untreated adherent cells	Colchicine-treated adherent cells	
1	97.5	97.7	91.8
6	93.0	NT[a]	88.3
20	80.3	NT	NT
29	16.9	NT	84.6
38	0	NT	NT
56	0	0	89.1

[a] Not tested.

CFA. Controls for both primary and secondary antisera and for medium all gave negative fluorescence.

With increasing age of the donor chickens, the percentage of CFA-positive adherent cells declined. Adherent cells derived from adult chickens (38 and 56 weeks of age) were found to be negative for CFA (Table I). While all assays were performed on adherent cell populations, the percentage of macrophage in the cell preparations was relatively constant (Table I). Therefore, the observed developmental loss of CFA from adherent cell populations cannot be due to variations in macrophage yield in test samples.

The relationship of CFA found on macrophage compared with that present on lymphocytes and erythrocytes is shown in Table II. R-anti-CFA adsorbed with 6-week bursacytes was negative against lymphocytes but retained reactivity against macrophage-rich adherent cells derived from chickens of the same age. This observation indicates the presence of ES–CFA determinants on macrophage. The fact that 6-week peritoneal macrophage bear different CFA determinants from both 1-day and 6-week erythrocytes can be seen from the LSCC-CU10 data (Table II). R-anti-CFA adsorbed to completion with LSCC-CU10 cells failed to react with adherent cells including macrophage but retained reactivity against peripheral erythrocytes. Apparently, macrophage bear some but not all of the ES–CFA determinants present on erythrocytes. Therefore, macrophage, bursacytes, and peripheral erythrocytes from 6-week-old chickens each bear unique combinations of cell surface CFA determinants.

Discussion. Little is presently known about cell surface markers on chicken macrophage. However, the finding that CFA is developmentally associated with activated peritoneal macrophage extends the developmental restriction of CFA to three hematopoietic dif-

TABLE II. CFA EXPRESSION ON HEMATOPOIETIC DIFFERENTIATION LINEAGES

Antisera	Test cells					
	1-day erythrocytes	LSCC-CU10	6-week bursacytes	6-week thymocytes	6-week macrophage	6-week erythrocytes
R-anti-CFA	+[a]	+	+	+	+	+
R-anti-CFA adsorbed w/bursacytes	NT[b]	+	−[c]	−	+	+
R-anti-CFA adsorbed w/ LSCC-CU10	+	−	NT	NT	−	+
R-anti-CFA inactivated by adsorption w/1-day chicken RBCs	−	−	−	−	−	−

[a] + indicates a positive reaction by microcytotoxicity (>50% above controls).
[b] Not tested.
[c] − indicates a negative reaction by microcytotoxicity (not greater than controls).

ferentiation lineages. The loss of CFA from adherent peritoneal exudate cells rich in activated macrophage exhibited a similar pattern to that previously reported for lymphocytes from secondary organs and peripheral erythrocytes. A transitional period exists when some, but not all, macrophage bear CFA. With increasing age, the CFA-positive population declines until only CFA-negative cells exist. This transition period for the macrophage apparently occurs somewhat later in development than previously observed for peripheral erythrocytes (16) and splenic lymphocytes (5). It is not known whether peripheral blood-derived macrophage from stimulated or unstimulated chickens would differ in CFA expression from the activated peritoneal macrophage used in the present study. Such a comparison must await further investigation.

While LA–CFA and ES–CFA were defined by previous work on lymphocytes, the present findings indicate that the macrophage differentiation lineage possesses a unique subset of CFA determinants different from LA–CFA and ES–CFA. While all three differentiation lineages possess CFA, the particular combination of CFA determinants present on each lineage can serve to identify the lineage. The significance of this observation lies in the nature of CFA. Since it has been proposed that CFA specificities are largely carbohydrate defined (9), lymphocytes, erythrocytes, and activated macrophage from the same aged chickens apparently possess microheterogeneity in the carbohydrate portion of CFA resulting in the observed differences in CFA expression. Since at least one CFA determinant has been shown to serve as a viral binding site (17), CFA heterogeneity between different hematopoietic differentiation lineages may have distinct biological significance.

One application of these findings will be the analysis of the differentiation status of avian tumor cells. CFA is known to occur on virally induced lymphoid tumors and related tumor cell lines (5, 18). Since specific differentiation lineage markers have now been identified, it will be possible to characterize these tumor cells according to their differentiation and developmental status of CFA expression (18). Therefore, CFA should be useful in determining the possible differentiation blockage or retrodifferentiation associated with specific tumorogenesis.

The authors thank Drs. M. Khalid Ashfaq, Bob Sanders, and Velibor Krsmanovic for their helpful discussions and Dr. K. A. Schat for donating the LSCC-CU10 cell line. The technical assistance of Dr. Lee Kass and Forrest Sanders and the editorial assistance provided by Dr. Margaret Dietert, Diane Wittner, and Diane Colf are greatly appreciated. This work was supported by NSF Grant PCM-81-09810 and NY(C) 157424 from the USDA.

1. Sanders BG, Dietert RR, Kline K, Dietert MF. Chicken fetal antigen: Example of an antigenically complex oncodevelopmental membrane glycoprotein. Oncodev Biol Med **2**:63–76, 1981.
2. Nelson CH, Patel NH, Sanders BG. Chicken fetal antigen expression on the definitive erythroid maturation series in the bone marrow of the adult chicken. J Exp Zool **212**:101–108, 1980.
3. Samarut J, Blanchet JP, Nigeon V. Antigenic characterization of chick erythrocytes and erythrocytic precursors: Identification of several definitive populations during embryogenesis. Dev Biol **72**:155–166, 1979.
4. Dietert RR, Lewin HA, Qureshi MA, Kass LB. Chicken fetal antigen: Association with lymphoid development and differentiation. J Exp Zool **202**:65–70, 1982.
5. Murthy KK, Dietert RR, Calnek BW. Demonstration of chicken fetal antigen (CFA) on normal splenic lymphocytes, Marek's Disease lymphoblastoid cell lines, and other neoplasms. Int J Cancer **24**:349–359, 1979.
6. Sanders BG. Fetal membrane antigens in normal development and neoplasia. In: Hildemann WA, ed. Frontiers in Immunogenetics. New York, Elsevier/North-Holland, 1981.
7. Dietert RR, Sanders BG. Expression of an onco-developmental antigen among avian species. J Exp Zool **206**:17–23, 1978.
8. Dietert RR, Lewin HA, Kass LB. Identification of a galactose-like component of a chicken oncodevelopmental antigen. J Hered **72**:257–260, 1981.
9. Lewin HA, Dietert RR. Characterization of a genetically segregating determinant of chicken fetal antigen by a new hapten inhibition of microcytotoxicity (HIM) assay. Biochem Genet **20**:425–436, 1982.
10. Cole RF, Hutt FB. Selection and heterosis in Cornell White Leghorns: A review, with special consideration of interstrain hybrids. Anim Breeding Abstr **41**(3):103–118, 1973.
11. Calnek BW, Murthy KK, Schat KA. Establishment of Marek's disease lymphoblastoid cell lines from transplantable versus primary lymphomas. Int J Cancer **21**(1):100–107, 1978.
12. Dietert RR, Sanders BG. Evidence for multiple cell surface chicken fetal-leukemic antigens (CFA) in the developing chick and other avian species. J Exp Zool **202**:171–177, 1977.
13. Desiderio JV, Campbell SG. Bovine mammary gland macrophage: Isolation, morphological features, and

cytophilic immunoglobulin. Amer J Vet Res **41**(10):1595–1599, 1980.

14. Sawer RT, Volkman A. Evaluation of monocyte distribution and traffic. In: Herscowitz HB, Holden HT, Bellanti JA, Ghaffar A, eds. Manual of Macrophage Methodology. New York, Marcel Dekker, Chap 52, pp459–469, 1981.
15. Lucas AM, Jamroz C. Atlas of Avian Hematology. Washington, DC, USDA Agr Monogr 25, 1961.
16. Dietert RR, Cirafesi J, Juran M. Developmental changes in the presence of chicken fetal-leukemic antigen determinants in the chicken, Japanese quail, and the interspecific hybrids. Poult Sci **59**:1706–1710, 1980.
17. Sanders BG, Wan KM, Kline K, Garry RF, Bose HR Jr. Chicken fetal antigen: Role as cell surface receptors for Sindbis virus hemagglutination. Virology **106**:183–186, 1980.
18. Dietert RR, Trembicki KA, Qureshi MA. Chicken fetal antigen as a differentiation marker on avian tumor cell lines. Oncodev Biol Med, in press, 1982.

Received June 14, 1982. P.S.E.B.M. 1983, Vol. 172.