主論文
初校

Detection of Epstein-Barr Virus RNA and Related Antigens in Non-Neoplastic Lymphoid Lesions.

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To elucidate the latent state and reactivation of Epstein-Barr virus (EBV) in non-neoplastic lymphoid lesions, we investigated 144 non-neoplastic lymphoid lesions by in situ hybridization (ISH) to detect the expression of EBV-encoded small RNAs (EBER)-1 and BCRF-1 and by immunostaining for latent membrane protein (LMP)-1 and ZEBRA. ISH for EBER-1 detected EBER-1-positive cells (EPCs) in 31 of the 144 examined lesions (22%). EPC were detected in 4 of 49 cases of nonspecific lymphoid hyperplasia, in 16 of 20 abscess-forming granulomatous lymphadenitis (AFGL), 5 of 25 Kikuchi's disease, and in 3 of 3 infectious mononucleosis. LMP-1 was expressed in 6 of 124 non-neoplastic lymphoid lesions (4.8%). LMP-1-positive cells were observed in 6 of the 31 EBER-1-positive cases (19%). EPCs were detected significantly more frequently in LMP-1- and ZEBRA-positive specimens than in the LMP-1- and ZEBRA-negative specimens. BCRF-1 was expressed in 4 of 11 cases examined; 2 of 3 AFGL, 1 of 2 Kikuchi's disease, and in the 1 case of atypical lymphoid hyperplasia. This study suggests that Epstein-Barr virus is prevalent and can be reactivated in the lymph nodes effaced by destructive inflammation, such as AFGL. Such inflammation may provide a local milieu that is conducive for EBV to enter the lytic cycle.

Key words: EBER-1, BCRF-1, LMP-1, ZEBRA, lymphoid lesion

Epstein-Barr virus (EBV) is involved in various neoplastic and non-neoplastic diseases, including infectious mononucleosis (IM) (1), nasopharyngeal carcinoma (2), Hodgkin's disease (3-7), non-Hodgkin's lymphoma (8-10), and some gastric cancers (11, 12). EBV has also been implicated in the etiology of autoimmune diseases such as Sjören's disease (13, 14) and rheumatoid arthritis (15). EBV initially infects epithelial elements of the oropharynx usually by salivary exchange, where the virus is in the lytic phase (16). Proliferating EBV infects B cells and persists there in a latent state (16, 17). Lymphoid tissues are thus postulated to act as reservoirs and to occasionally supply EBV particles to epithelial elements by reactivation, infecting new hosts (17, 18). Lymphokines and stimulatory agents such as phorbol ester reactivate EBV in vitro (16). However, the way in which EBV is reactivated *in vivo* is still unclear. Most studies have focused on EBV in malignant tumors. However, EBV infection in non-neoplastic lesions, apart from IM, has been reported in only a few studies (19-22). We believe that investigation in EBV in non-neoplastic lymphoid lesions may help our understanding of the life cycle of this virus, particulary the conditions under which it is reactivated.In this study, we investigated EBV in non-neoplastic lymphoid lesions by in situ hybridization (ISH) to detect EBV-encoded small RNAs (EBER)-1 and BCRF-1 expression and by immunostaining to detect latent membrane protein (LMP)-1 and ZEBRA. ISH for EBER-1 is a useful method for detecting latent EBV infection (23). LMP-1 promotes the proliferation of EBV-infected cells, but it is a target of cell-mediated immunity. It cannot be expressed without rejection *in vivo* unless the local immunological milieu is destroyed, as occurs in Hodgkin's disease and in AIDS-related lymphoma. We therefore used it to survey the local suppression of cell-mediated immunity. ZEBRA, one of the immediate early proteins, is encoded in BZLF-1 (24, 25). ZEBRA acts as a switch protein between latency and

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replication of EBV. During latency of EBV, ZEBRA expression is repressed. Inducing stimuli cause the synthesis of ZEBRA which, in turn, activates expression of several early genes. Thus, ZEBRA is a site-specific DNA protein that likely functions as a transcriptional transactivator (16). BCRF-1 (vIL-10), recently identified as a latency gene (26), is a homologue of hIL-10; hIL-10 modulates the immune response by suppressing the synthesis of cytokines in Th1, $CD8^+$ T lymphocytes, NK cells, and macrophages by inhibiting the accessory cell effector functions of macrophages (27). vIL-10 is thought to act as an immunosuppressant *in vivo*.

Material and Methods

Case selection. We selected 144 cases of benign lymphoid lesions from the files of the Department of Pathology at our institution; 49 were non-specific lymphoid hyperplasia; 20, abscess-forming granulomatous lymphadenitis (AFGL); 25, Kikuchi's disease; 9, dermatopathic lymphadenitis; 9, tuberculous lymphadenitis; 3, IM; 5, atypical lymphoid hyperplasia; 20, perigastric lymph nodes associated with gastric cancer, and 4 other lesions (1 Kimura's disease, 1 idiopathic plasmacytic lymphadenopathy, 1 inflammatory pseudotumor, and 1 angiolymphoid hyperplasia). In the patients with atypical lymphoid hyperplasia, histological findings were suggestive of malignancy, but neither immunogenotypic studies nor the clinical courses showed a malignant nature. EBV serologic data were available only for 2 patients, both of whom had IM. All tissues were fixed in formalin solution and paraffin-embedded. Tissue sections were mounted on glass slides that had been pretreated with 3-aminopropyltriethoxysilane (Matunami, Tokyo, Japan) and were baked for 2 hr at 47°C and stored at 4°C until use.

In situ hybridization. Paraffin sections were subjected to ISH to detect EBER-1 and BCRF-1 expression. After deparaffinization, the sections were treated with 0.2 N HCl for 10 min, 5 μg/ml proteinase K in 50 mM Tris-HCl (pH 8.0) at 37°C for 15 min, and 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. For the detection of EBER-1, an 0.5 ng/μl FITC-labeled antisense EBER-1 oligonucleotide probe, described by Chang et al (14). (5'-AGACACCGTCCTCACC ACCCGGGACTTGTA-3'), was applied to the specimens and hybridized at 37°C overnight. As a control probe, we used a 30-base-pair oligonucleotide (5'-ACAGAGGCTGGTGAGGAGGGCCCACGTGTA-3') labeled with FITC. After hybridization, the probes were washed in Tris-buffered saline (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.1% Tween 20. Detection was performed with an in situ detection kit, KO46 (Dakopatts, Glostrup, Denmark). Positive signals were visualized as dark blue or black staining on nuclei. The number of EBER-1-positive cells (EPC) per 1 cm^2 of lesion was counted. More than 5 EPC in a high-power field in a lesion was termed a cluster. To detect BCRF-1 expression, we used a digoxigenin-labeled RNA probe. The part of the BCRF-1 gene (9680-10195), which showed low homology to the human IL-10 gene, was determined by using a gene analysis software (GENETYX-MAC) and used as a probe. The DNA fragment of BCRF-1 amplified from B95 was packed in PCR™ (Invitrogen, Leek, Netherlands). The digoxigenin-labeled sense and anti-sense probes were generated using a digoxigenin RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Pretreatment was carried out as described above, followed by the application of RNA probes in hybridization buffer (2 ng/ml). After overnight hybridization at 45°C, the slides were washed once in 2X SSC at room temperature, incubated in 2X SSC containing 50% formamide at 45°C for 30 min, and then washed twice in 2X SSC at room temperature. They were then treated with 5 mg/ml RNase for 30 min at room temperature and visualized with a DNA detection kit (Boehringer Mannheim).

Immunohistochemistry. For LMP-1, the sections were treated with 0.1% trypsin for 30 min and for ZEBRA they were autoclaved in citrate buffer. Then, they were immersed in 3% H_2O_2 in PBS for 5 min to block endogenous peroxidase activity, and soaked in the serum of an individual not infected with EBV; the serum was diluted with PBS to 10%. Immunostaining was performed by the avidin-biotin peroxidase complex (ABC) method, using anti-LMP-1 monoclonal antibody (mAb) (1:50, Dakopatts) and anti-ZEBRA mAb (1:30, Dakopatts). Specimens of Hodgkin's disease and B95-8 cells (EBV-infected marmoset cell line) were used as the positive controls for LMP-1 and ZEBRA, respectively.

Polymerase chain reaction (PCR) assay. DNA was extracted from the paraffin-embedded sections of selected cases. DNA samples (1 μg) were subjected to 30 amplification cycles (30 sec at 95°C, 30 sec at 50°C, and 2 min at 72°C) in a DNA thermal cycler (MJ Research, Watertown, USA), in a total volume of 100 μl

PCR mixture, containing 3 U of *Tth* DNA polymerase (TOYOBO Biochemicals, Tokyo, Japan). The amplified product was electrophoresed in 3% NuSieve gel (Bio Rad, Hercules, USA) and photographed. The sequences of the primers were: TC60, CCAGAGGTAAGTGGACTT; TC61, GACCGGTGCCTTCTTAGG (28).

Statistical analysis. The significance of differences in the number of EPC per cm2 in each pathological specimen was analyzed by the Kruskal-Wallis test. The number of EPC of the two groups was compared by the Mann-Whitney U-test.

Results

EBER-1 expression. EPC were demonstrated by ISH in 31 of the 144 cases of non-neoplastic lymphoid lesions: 16 of 20 AFGL, 4 of 49 non-specific lymphoid hyperplasia, 5 of 25 Kikuchi's disease, 1 of 9 tuberculous lymphadenitis, 3 of 3 IM, and 2 of 5 atypical lymphoid hyperplasia (Table 1). EBER-1 expression was observed on both small lymphocytes and blastic cells (Fig. 1). The number of EPC in each lesion ranged from 2 to 9700 / cm^2 (Table 3). AFGL, IM, and Kikuchi's disease contained a significant large numbers of EPC ($P<0.001$). EPC in IM and AFGL were numerous and outnumbered those in Kikuchi's disease ($P<0.001$). In AFGL, EPC were found in the interfollicular areas around the abscesses and were rarely seen in the lymphoid follicles. They were not scattered uniformly and tended to cluster in small areas.

BCRF-1 expression. BCRF-1 expression was examined in 11 cases: 3 of AFGL, 3 of Kikuchi's disease, 1 of atypical lymphoid hyperplasia, and 4 of non-specific lymphoid hyperplasia. BCRF-1 was detected

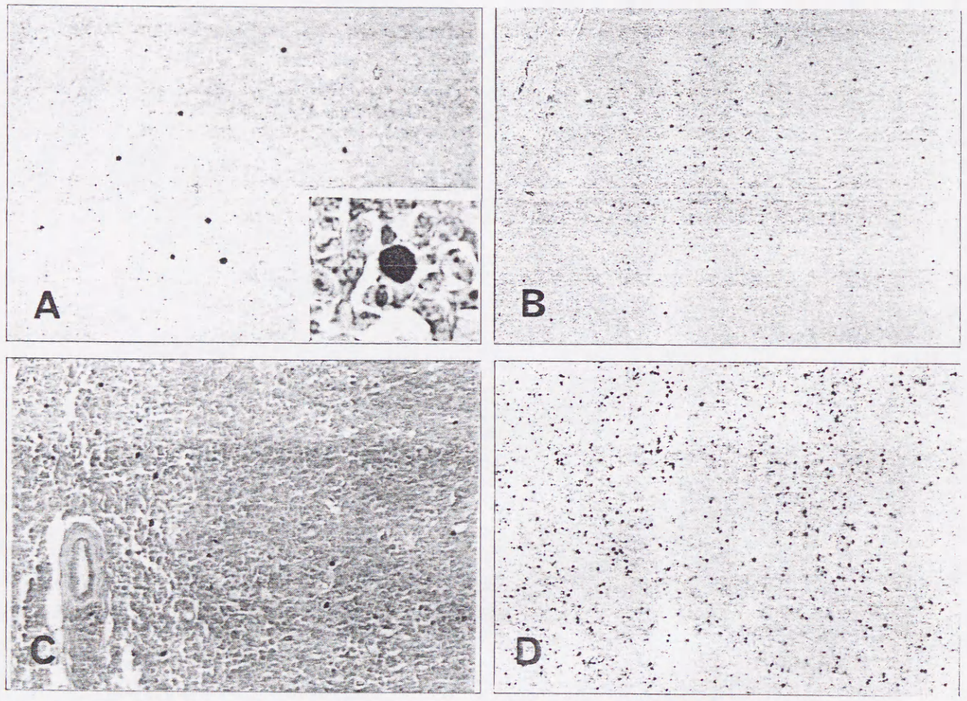


Fig.1 Expression of EBER-1 in non-neoplastic lymphoid lesions. *In situ* hybridization. A: Kikuchi's disease (× 100). B: Abscess-forming granulomatous lymphadenitis (× 40). C: Atypical lymphoid hyperplasia (× 100). D: Infectious mononucleosis (× 40). Note the black staining in the nuclei. Inset: EBER-1-positive blastic lymphoid cell (× 1000).

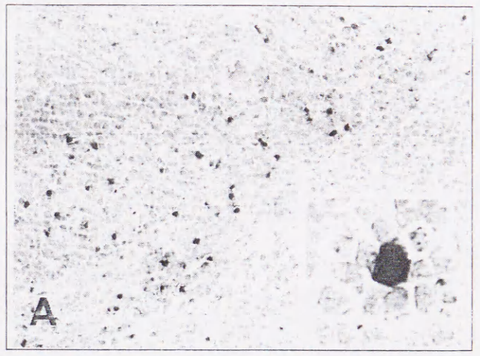
Table 1 Detection of EBER-1 expression by in situ hybridization

Disease	No. of cases	No. of EBER-1-positive cases	%
Non-specific lymphoid hyperplasia	49	4	(8)
Abscess-forming granulomatous lymphadenitis	20	16	(80)
Kikuchi's disease	25	5	(20)
Dermatopathic lymphadenitis	9	0	(0)
Atypical lymphoid hyperplasia	5	2	(40)
Tuberculous lymphadenitis	9	1	(11)
Infectious mononucleosis	3	3	(100)
Perdgastric lymph node associated with gastric cancer	20	0	(0)
Other[a]	4	0	(0)
Total	144	31	(22)

a: Kimura's disease, idiopathic plasmacytic lymphadenopathy, inflammatory pseudotumor, and angiolymphoid hyperplasia

in 2 cases of AFGL, 1 case of Kikuchi's disease, and 1 case of atypical lymphoid hyperplasia. BCRF-1 was expressed on lymphoid cells including blasts (Fig. 2). The number of BCRF-1-positive cells was less than the number of EPC observed in the same cases.

LMP-1 and ZEBRA expression. LMP-1 was expressed in 6 of 124 cases (including the 31 that were EBER-1-positive) (Tables 2, 3); it was expressed in 2 of 20 cases of AFGL, 2 of 5 cases of atypical lymphoid hyperplasia, and 2 of 3 cases of IM. All 6 of these LMP-1-positive cases were also EBER-1-positive. LMP-1-positive lymphocytes were scanty in number except for one case of IM (case 27) which contained many LMP-1-positive blastic cells including some small ones (Fig. 3A). ZEBRA was examined in the same 124 cases (Fig. 3B) and was observed in 13; 3 cases of IM, 8 of AFGL, 1 of Kikuchi's disease, and 1 of atypical lymphoid hyperplasia. The density of EPC in ZEBRA-positive cases was significantly higher than that in ZEBRA-negative cases.



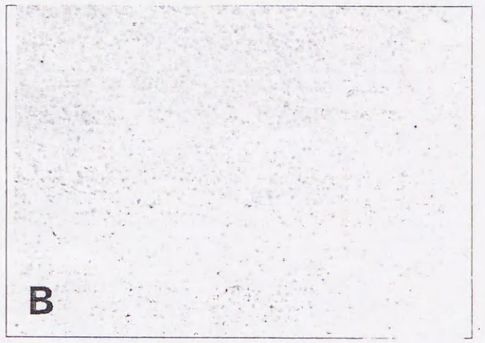


Fig.2 Expression of BCRF-1 in abscess-forming granulomatous lymphadenitis (case 6). *In situ* hybridization. A: Anti-sense probe. BCRF-1 expression was detected in the nuclei of some cells (X 200). B: Sense probe (X 200). Inset: BCRF-1-positive blastic lymphoid cell (X 1000).

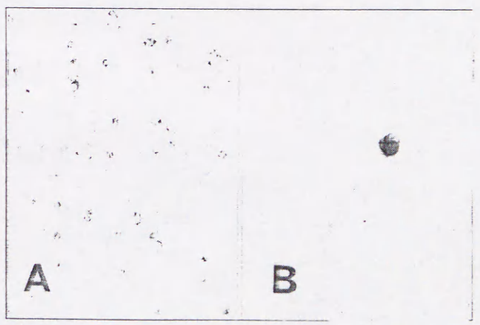


Fig.3 Immunohistochemical detection of LMP-1 and ZEBRA A: LMP-1, IM (case 27) (X 200). B: ZEBRA, ALH (case 30) (X 400).

Table 2 Summary of EBER-1-positive cases

Case	Disease	EPC	Cluster	LMP	ZEBRA	PCR	BCRF-1
1	NSLH	28	−	−	−	ND	−
2	NSLH	16	−	−	−	−	−
3	NSLH	10	−	−	−	−	ND
4	NSLH	2	−	−	−	−	ND
5	AFGL	24	−	+	−	ND	ND
6	AFGL	630	+	−	+	+	+
7	AFGL	256	+	+	+	+	+
8	AFGL	38	+	−	−	+	−
9	AFGL	2502	+	−	+	+	ND
10	AFGL	1134	+	−	+	+	ND
11	AFGL	440	+	−	+	−	ND
12	AFGL	16	−	−	−	−	ND
13	AFGL	12	−	−	−	−	ND
14	AFGL	122	+	−	+	+	ND
15	AFGL	264	+	−	−	−	ND
16	AFGL	352	−	−	+	+	ND
17	AFGL	242	+	−	−	−	ND
18	AFGL	11	−	−	−	−	ND
19	AFGL	4500	+	−	+	−	ND
20	AFGL	16	−	−	−	−	ND
21	KD	8	−	−	−	ND	ND
22	KD	12	−	−	−	−	+
23	KD	125	−	−	+	−	ND
24	KD	2	−	−	−	−	ND
25	KD	160	+	−	−	−	−
26	Tbc.L	5	−	−	−	ND	ND
27	IM	9700	+	+	+	ND	ND
28	IM	4800	+	+	+	ND	ND
29	IM	8300	+	−	+	ND	ND
30	ALH	632	+	+	+	+	+
31	ALH	112	−	+	−	−	ND

NSLH: Non-specific lymphoid hyperplasia
KD: Kikuchi's disease
IM: Infectious mononucleosis
ND: Not done
AFGL: Abscess-forming granulomatous lymphadenitis
Tbc.L: Tuberculous lymphadenitis
ALH: Atypical lymphoid hyperplasia

Table 3 Immunohistochemical detection of LMP-1 and ZEBRA

Disease	No. of cases	LMP-1	%	ZEBRA	%
Non-specific lymphoid hyperplasia	49	0	(0)	0	(0)
Abscess-forming granulomatous lymphadenitis	20	2	(10)	8	(40)
Kikuchi's disease	25	0	(0)	1	(4)
Dermatopathic lymphadenitis	9	0	(0)	0	(0)
Atypical lymphoid hyperplasia	5	2	(40)	1	(20)
Tuberculous lymphadenitis	9	0	(0)	0	(0)
Infectious mononucleosis	3	2	(67)	3	(100)
Other[a]	4	0	(0)	0	(0)
Total	124	6	(5)	13	(11)

a: See footnote of Table 1.

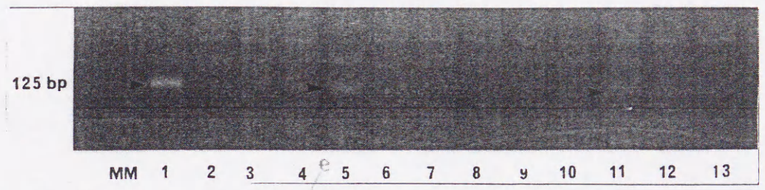


Fig.4 Detection of Epstein-Barr viral genome by polymerase chain reaction. *Bam*HI W fragments were amplified. MM indicates molecular marker (Funakoshi, BioMarker Low). Lane 1, Raji (EBV-infected cell line), positive control; lanes 2-4, non-specific lymphoid hyperplasia; lane 5, abscess-forming granulomatous lymphadenitis; lanes 6-10, Kikuchi's disease; lane 11, atypical lymphoid hyperplasia; lanes 12-13, dermatopathic lymphadenitis. Arrows show the amplified PCR products (123 bp).

Detection of EBV genome by polymerase chain reaction. PCR was carried out in 34 cases: 6 non-specific lymphoid hyperplasia (3 EBER-1$^+$ and 3 EBER-1$^-$), 17 AFGL (15 EBER-1$^+$ and 2 EBER-1$^-$), 7 Kikuchi's disease (4 EBER-1$^+$ and 3 EBER-1$^-$), 2 dermatopathic lymphadenitis (EBER-1$^-$), and 2 atypical lymphoid hyperplasia (EBER-1$^+$). The EBV genome was detected in only 8 cases (7 of AFGL and 1 of atypical lymphoid hyperplasia) (Fig. 4). These EBV genome-positive cases also expressed EBER-1 (Table 3).

Discussion

In this study, we examined EPC in various non-neoplastic lymphoid lesions. All the IM cases examined contained a large number of EPC. AFGL and Kikuchi's disease sections contained a significantly larger number of EPC than sections from the other lesions except for IM. In both AFGL and Kikuchi's disease, the lymph nodes are effaced by abscesses or necrosis and this obliteration of the structure may be responsible for the high density of EPC. However, the mean number of EPC and the proportion of EBER-positive cases in AFGL were greater than those in Kikuchi's disease, and tuberculous lymphadenitis, showing massive caseous necrosis, contained fewer EPC than Kikuchi's disease; these findings suggest that necrosis alone may not provide a favorable milieu for the reactivation of EBV. We speculate that the suppurative infection in AFGL provides a better milieu for the increase of EPC than the non-suppurative necrosis in Kikuchi's disease or the caseous necrosis in tuberculosis. The presence of numerous polymorphs and secreted cytokines in AFGL may be contribute to the reactivation of EBV. The number of EPCs was greater in ZEBRA-positive than in ZEBRA-negative cases ($P<0.01$), suggesting that EPC increase when EBV is reactivated. Lytic infection is a rare event in EBV-infected malignant lymphomas (5, 6, 10, 29). However, we found here that lytic infection was frequent in non-neoplastic lesions with more than 100 EPC/cm^2. This finding implies that EBV in non-neoplastic lesions behaves in a different manner from EBV in malignant lymphoma.The EBV genome was detected in only 8 of 34 cases examined by PCR, which included 24 EBER-1$^+$ cases. This incidence of PCR positivity is much lower than that of EBER-ISH. This higher sensitivity of EBER-ISH is consistent with the results reported previously (4, 29), and may be due to a very high copy number (up to 10^6) of EBER transcripts in the nucleus of latently infected cells (30).LMP-1 expression was observed in only 6 of 116 cases. Of these 6 cases, 4 contained more than 100 EPC/cm^2, i.e., the number of LMP-1-positive cells was much lower than the number of EPC. This low number of cells expressing LMP-1, a protein that is a target of cell-mediated immunity, appears to reflect the destruction of these cells by host immune surveillance. On the contrary, however, LMP-1 expression also reflects the suppression of local immunological surveillance in the lymph node. These observations suggest that the increase of EPC in non-neoplastic lesions is related to the suppression of local immunological surveillance. We detected ZEBRA expression in 13 of 116 cases and reactivation of EBV was found only in those cases with a high density of EPC. This suggests that the number of EPC is increased by reactivation *in vivo*. BCRF-1 expression was detected in 4 of 11 examined cases. We observed ZEBRA-positive cells in 3 of these 4 BCRF-1-positive cases, but not in any of the 7 BCRF-1-negative cases. This implies that BCRF-1 suppresses immunosurveillance during reactivation, as has already been suggested (26). Our findings in this study are consistent with the hypothesis that BCRF-1 may have a role in the interaction of the

virus with the host immune system, participating in the escape of virus-infected B cells from T-cell surveillance (26,31). Dissemination of the replicated virus may be an important step in the early development of EBV-associated malignancy. Investigation of the functions of the viral products involved in reactivation *in vivo* is now the focus of EBV research (16, 29, 32).

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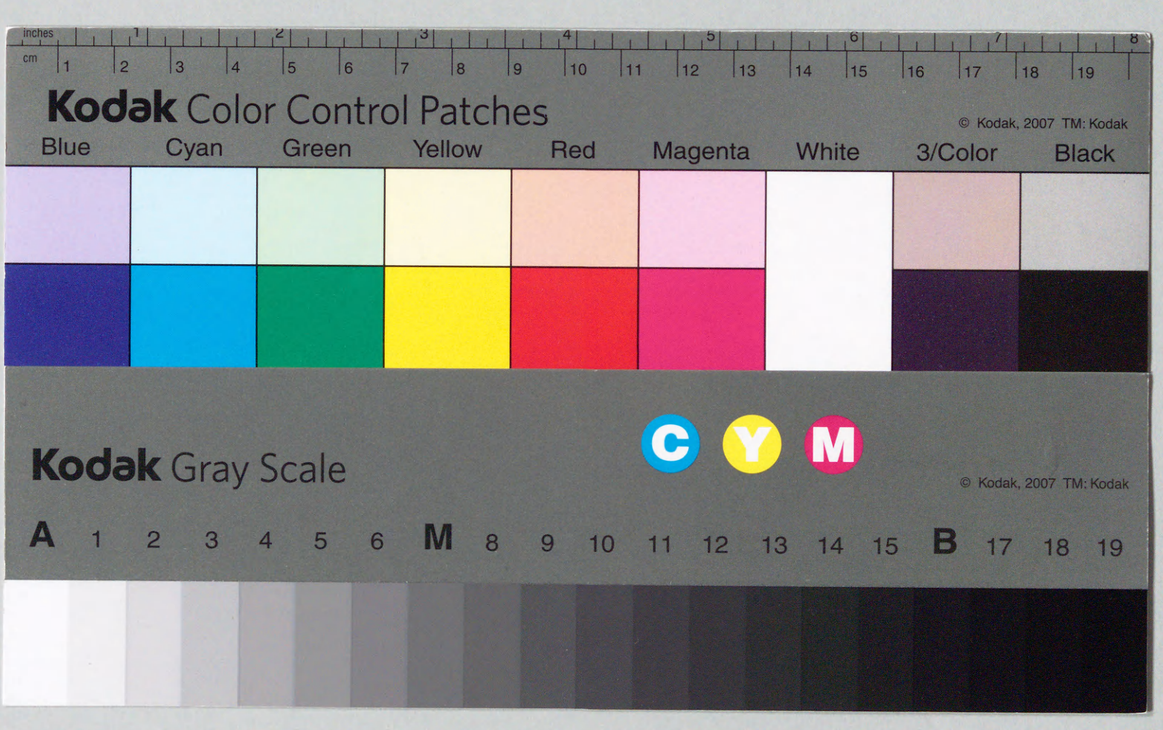
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